Structure and reorientational dynamics of angiotensin I and II: a microscopic physical insight

Kristi Y. DeLeon¹, Achal P. Patel¹, Krzysztof Kuczera², Carey K. Johnson³ and Gouri S. Jas*⁴

¹Department of Chemistry, Biochemistry, Institute of Biomedical Studies, Baylor University, Waco, TX 76706, USA; ²Department of Chemistry and Department of Molecular Biosciences, University of Kansas, Lawrence, KS 66045, USA

Communicated by Ramaswamy H. Sarma

(Received 15 July 2011; final version received 15 October 2011)

We present a study of structural analysis and reorientational dynamics of Angiotensin I (AngI) and Angiotensin II (AngII) in aqueous solution. AngI is a decapeptide that acts as a precursor to the octapeptide AngII in the Renin–Angiotensin–Aldosterone system for blood pressure regulation. Experimental structural characterization of these peptides, carried out with circular dichroism and infrared spectroscopy, showed that the angiotensins are mostly disordered but exhibit a measurable population of ordered structures at room temperature. Interestingly, these change from the unordered polyproline-like conformation for AngI to a more compact and ordered conformation for AngII as the length of the peptide is decreased. Anisotropy decay measurements with picosecond time resolution indicate slower overall tumbling and a greater amplitude of internal motion in AngI compared to AngII, consistent with more compact and less flexible structure of the active form of the peptide. To model the microscopic behavior of the peptides, 2-μs molecular dynamics simulation trajectories were generated for AngI and AngII, at 300 K using the OPLS-AA potential and SPC water. The structures sampled in the simulations mostly agree with the experimental results, showing the prevalence of disordered structures, turns, and polyproline helices. Additionally, the computational results predict fewer sampled conformations, tighter side-chain packing and marked increase of Phe8 solvent accessibility upon AngI truncation to AngII. Our combined approach of experiment and extensive computer simulation thus yields new information on the conformational dynamics of the angiotensins, helping provide insight into the structural basis for the potency of AngI relative to AngII.

Keywords: Angiotensin; circular dichroism; FTIR; anisotropy decay; reorientational dynamics; molecular dynamics

Introduction

The Renin–Angiotensin–Aldosterone system is the most important signaling cascade that regulates blood pressure through monitoring blood volume and sodium/fluid balance. According to the World Health Organization, hypertension is estimated to cause 7.1 million worldwide deaths annually, or 13% of total deaths, and 4.4% of the total disease burden (Puska, Mendis, & Porter, 2003). It is estimated that hypertension will cost the USA about $77 billion in 2010 alone (Lloyd-Jones, Adams, & Carnethon, 2009). Patients diagnosed with hypertension often develop complications such as cerebrovascular disease, cardiovascular disease, peripheral vascular disease, and renal failure (McManus, Freel, & Connell, 2007). Because of these fatal complications, hypertension is often referred to as the “silent killer” (Chilman-Blair & Rabaseda, 2003). A drop in blood pressure, a decrease in circulating blood volume, or a reduction in plasma sodium concentration stimulates the juxtaglomerular cells of the kidneys to release renin, an enzyme that cleaves the serum protein precursor Angiotensinogen into Angiotensin I (AngI) (Chilman-Blair & Rabaseda, 2003; Solomon & Anavekar, 2005). AngI has no known effect on the body and may act solely as a precursor to Angiotensin II (AngII). The formation of AngII is mainly by the action of Angiotensin Converting Enzyme (ACE). ACE is a halide-requiring peptidase that catalyzes the cleavage of the carboxyl end of the decapeptide AngI (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) to form the vasopressor, AngII (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) (Solomon & Anavekar, 2005).

AngII is a potent vasoconstrictor (Chilman-Blair & Rabaseda, 2003), stimulating the sympathetic constriction of the circulatory and renal vasculature, hormone secretion by the anterior and posterior pituitary and adrenal glands, and promotion of renal conservation of sodium and water (Cho & Asher, 1996). There are four types of angiotensin receptors of which only two, AT₁ and AT₂, bind AngII despite their low degree of sequence homology (34%) (Chilman-Blair & Rabaseda, 2003; Oliveira et al., 2007). AT₁ receptors mediate the majority of AngII’s physiological actions including regulation of blood pressure, water, and electrolyte balance. The physiological role of the AT₂-receptor is still not clearly understood.
understood though it is believed to have a significant role in apoptosis, tissue repair, and fetal development (Deraet et al., 2002). The pathogenesis of cardiovascular disease such as hypertension, cardiac hypertrophy, and congestive heart failure is believed to be associated with prolonged angiotensin stimulation (Aplin et al., 2007). In heart muscle, when AngII binds to its receptors AT\textsubscript{1} and/or AT\textsubscript{2}, it stimulates nicotinamide adenine dinucleotide phosphate oxidase (NOX) activation and superoxide generation, which causes the induction of apoptosis and fibrosis. The resulting apoptosis contributes to cardiomyopathy development (Oliveira et al., 2007).

Understanding the structure of AngII when bound to the receptor is important for determining the ligand–receptor interaction that is necessary for receptor activation. Previous studies have shown the binding site of AngII to be within both the receptor’s extracellular loops and transmembrane domains (Garcia, Desiderio, Ronco, Verroust, & Amzel, 1992; Spyroulias et al., 2003; Tzakos, Gerothanassis, & Troganis, 2004; Yamano et al., 1995). In order to mimic receptor bound AngII, Garcia et al., used X-ray crystallography on monoclonal antibody-bound (MAb) AngII and discovered a compact conformation with two turns located between residues Ile5-His6-Pro7 and Asp1-Arg2 (Garcia et al., 1992). Modeling studies performed with the AT\textsubscript{1}-receptor have shown AngII to be bound in a U-shape conformation (Spyroulias et al., 2003; Tzakos et al., 2004; Yamano et al., 1995), which agreed with the MAb AngII structure. NMR results of aqueous AngII have also shown AngII in a U-shaped conformation with a turn located between Ile5-His6-Pro7 residues (Spyroulias et al., 2003; Tzakos et al., 2004). In AT\textsubscript{2}-receptor molecular modeling studies, positioning of previously suggested ligand–receptor contacts resulted in AngII adopting a β-extended conformation that extends into the hydrophobic pocket (Boucard et al., 2000; Deraet et al., 2002; Sköld, Nikiforovich, & Karlén, 2008). AngII has been studied using various spectroscopic techniques, but the interpretation of the conformational studies has been inconsistent, most likely from solvent structural dependence, which has led to various structural models for AngII in solution including β pleated sheet, β and γ turns, and other structures (Spyroulias et al., 2003; Tzakos et al., 2004). Small linear peptides normally exist in solution as a range of conformations in rapid equilibrium, which may explain the variation of structural predictions in the numerous studies of AngII (Matsoukas, Bigam, Zhou, & Moore, 1990).

Due to increases in computer power, full conformational sampling with direct molecular dynamics simulations has become possible for small and medium-sized peptides in aqueous solution. Systems studied previously include a beta-hexapeptide (Daura et al., 2001), CW1 and CW2 peptides (Yeh & Hummer, 2002), and penta-alanine (Buchete & Hummer, 2008). Using distributed computing, the folding kinetics of the 21-residue Fs peptide has been analyzed (Sorin & Pande, 2005). More recently, both equilibrium and kinetics of folding of the blocked penta-alanine have been simulated using standard modeling tools (Hegefeld, Chen, DeLeon, Kuczera, & Jas, 2010), while folding of several small proteins was followed on a specialized computer system (Piana, Lindorff-Larsen, & Shaw, 2011). Such simulations provide a wealth of microscopic information on peptide and protein behavior, greatly increasing our understanding of the observed properties of these interesting systems.

In this present study, we describe a combined experimental and computational investigation aimed at providing a detailed picture of the conformational and dynamic behavior of AngI and AngII peptide systems. Experimentally, far-UV circular dichroism (CD) spectroscopy and Fourier transform infrared spectroscopy were employed to characterize the structures of AngI and AngII and fluorescence anisotropy decay measurements with picosecond time resolution were used to describe dynamics of peptides in the solution at physiological pH and acidic conditions. Compared to previous structural studies that indicated that AngII exists in a turn-like conformation, our results suggest the presence of more compactly defined structural elements. Our results show that the angiotensins are mostly disordered, but exhibit a measurable population of ordered structures: polyproline helix for AngI and more compact, very possibly a left-handed helix and extended for AngII. In terms of dynamics, the peptides undergo conformational fluctuations at 40–100 ps and overall tumbling at 400–600 ps timescales. The results indicate a more compact and rigid average structure for AngII. Computationally, we performed 2-μs molecular dynamics (MD) simulations for the peptides with the OPLS-AA potential and SPC water. The structures and motions in the simulations mostly agree with the experimental results, showing the prevalence of disordered structures, turns and polyproline helices, the more compact average structure of AngII relative to AngI, and reasonable dynamical rates. Additionally, the computational results predict fewer sampled conformations, tighter side-chain packing, and marked increase of Phe8 solvent accessibility upon AngI truncation to AngII. Our combined approach of experiment and computer simulation thus shows that AngII, the active form of angiotensin, resides in a more compact well-defined secondary structural conformation, as verified with far-UV CD and anisotropy measurements, helping provide insight into functional behavior of AngI and AngII.

**Methods**

**Experiment**

**Far-UV CD spectroscopy**

The peptides AngI and AngII were purchased from GenScript Corporation (Piscataway, NJ, USA) and were >99% pure. Temperature dependent far-UV CD measurements were performed with a Jasco 815 spectropolarimeter (Tokyo, Japan) in both 20 mM acetate buffer at pH 4.8 and 20 mM phosphate buffer at pH 7.2. The sample concentrations were all about 500 μM. CD was performed as a function of temperature (267–363 K) in a
Fourier transform infrared spectroscopy

FTIR measurements at 298 K were performed on AngI and AngII in both 20 mM acetate buffer pH 4.8 and 20 mM phosphate buffer pH 7.2 with concentrations of around 1 mM. The samples were measured with CaF$_2$ windows separated by a 15 µm Teflon spacer. Measurements were collected from 400–4000 cm$^{-1}$ with a resolution of 1 cm$^{-1}$ and 10,000 scans using a Thermo Nicolet Nexus 670 FT-IR equipped with a KBr beamsplitter and DTGS KBr detector. The buffer solution corresponding to the sample was interactively subtracted using the OMNIC software (Thermo Electron Corporation). Data analysis was performed with OMNIC software utilizing Fourier self-deconvolution (FSD) and curve-fitting procedures using least square fits with gaussian profiles.

Fluorescence anisotropy decay measurements

The time-resolved fluorescence studies were carried out by time-correlated single-photon counting. Fluorescence was excited at 280 nm with the third-harmonic of a mode-locked, cavity-dumped Mira Optima 900F/Pulse Switch Ti:Sapphire laser pumped by a 10 W Verdi Laser (Coherent, Inc., Santa Clara, CA, and 5-050 Ultrafast Harmonic Generator, Inrad Northvale, NJ). Fluorescence was collected at 307 nm with an 8-nm bandpass (model 9030 monochromator, Sciencetech Inc., Concord, ON, Canada). Parallel and perpendicular fluorescence polarizations were collected simultaneously in a T-format system described in detail elsewhere (Unruh, 2006) and processed by a PC card (Becker and Hickl, SPC-830, Berlin, Germany). Fluorescence decays were measured for samples in both 20 mM acetate buffer pH 4.8 and 20 mM phosphate buffer pH 7.2 at 283 K, 293 K, and 303 K. Fluorescence decays for parallel and perpendicular polarizations were fit globally with in-house software to a three-exponential intensity decay $I(t)$ coupled with a double-exponential anisotropy decay $r(t)$:

$$I_x(t) = I(t)[1 + 2r(t)]$$

$$I_y(t) = G \cdot I_x(t)[1 - r(t)]$$

where the factor $G$ corrects for the different transmission and detection efficiencies in the two detection channels ($G \approx 1.5$). The instrument response function had a full-width-half-maximum (FWHM) of $\sim$50 ps.

MD simulations

MD simulations were carried out for four systems, ANGln – angiotensin I with neutral forms of His6 and His9, ANGIlp – angiotensin I with protonated His6 and His9, ANGIn – angiotensin II with neutral His6 and ANGIIlp – angiotensin II with protonated His6. ANGln and ANGIn correspond to peptides at neutral pH, while ANGIlp and ANGIIlp represent states expected at lower pH. In each case the peptide was built in extended form using the CHARMM program (Brooks et al., 2009), placed in a cube of water molecules, and Na$^+$ and Cl$^-$ counterions were added to neutralize the system at close to physiological ionic strength (see Table 1 for system description). Standard termini were employed in all cases, i.e. –NH$_2$ at N-terminus and –COO$^-$ at C-terminus. Each system underwent a brief energy minimization, 20 ps of solvent equilibration with position restraints on solute and 100 ps of full NPT equilibration at 1 atm and 300 K. Finally, NVT trajectories of 2 µs length were generated at 300 K, using the GROMACS 4.0.4 code (Hess, Kutzner, van der Spoel, & Lindahl, 2008), running in parallel on 16 nodes. We used the OPLS-AA/L (Kaminski, Friesner, Tirado-Rives, & Jorgensen, 2001) force field for the peptides and the SPC water model (Berendsen, Postma, van Gunsteren, & Hermans, 1981), and the Particle-Mesh Ewald (Saigui & Darden, 1999) (PME) method for long-range electrostatics. Van der Waals interactions were truncated at 14 Å, a time step of 2 fs was employed with all bond lengths constrained.

Analysis

The basic calculated quantities were the backbone atom root-mean-square deviation (RMSD) from target structures, the end-to-end distances, radii of gyration, distances between centers of mass of selected residues, solvent accessible surface areas, and the values of the backbone ($\phi$, $\psi$) dihedral angles of the central residues (2–9 for AngI and 2–7 for AngII). Conformation clustering was performed using the GROMOS algorithm (Daura, Gademann, Jaun, & Mark, 1999), using a set of 50,000 structures sampled every 40 ps from each trajectory. For each structure in the pool, the number of neighbors within a cutoff distance are counted, and the structure with largest number of neighbors, together with these neighbors, assigned to a cluster. This cluster is eliminated from the pool and analysis is repeated (Daura et al., 1999). Clustering was based on RMSD, using a radius of 3 Å and both backbone and all non-H atoms. Secondary structure analysis was done using the DSSP algorithm (Kabsch & Sander, 1983). An additional characterization of the backbone conformations was based on the Ramachandran map of residue backbone ($\phi$, $\psi$). Four main regions were defined: $\alpha_R$, centered at ($-60^\circ$, $-40^\circ$), $\alpha_L$, centered at ($+60^\circ$, $+40^\circ$), polyproline II (PPII), centered at ($-80^\circ$, $150^\circ$), and $\beta$, centered at ($-140^\circ$, $135^\circ$). To capture a majority of structures of the flexible angiotensins into these four categories, conformers were assigned to a basin if they were within a $60^\circ$ radius of one of these four central points. As a consequence of the large radius size, this is quite a coarse analysis, e.g. the “$\alpha_R$” basin encompasses $\alpha$, 3–10 and $\pi$ helices as well as other structures in the lower left quadrant of the ($\phi$, $\psi$) map (and similarly for “$\alpha_L$”), and the PPII and $\beta$ basins.
exhibit partial overlap, which leads to some structures being counted twice.

The fluorescence anisotropy decay experiments were modeled by calculating the correlation function $C_2(t)$ of the transition dipole vector of the Tyr4 ring, $C_2(t) = (3\cos^2\theta(t) - 1)/2$, where $\theta(t)$ is the axis reorientation angle during time $t$. The trajectory average over starting structures and the transition dipole is along the short in-plane axis of the ring (Kuczera, Unruh, Johnson, & Jas, 2010). This correlation function is proportional to the anisotropy $r(t)$ and its decay components may be directly compared to experimental data (Park, Shalongo, & Stellwagen, 1997). To provide a description of the overall peptide tumbling, we also calculated $C_2(t)$ for the end-to-end vector, i.e. the vector connecting the fist to last alpha-carbon. To determine time constants for motion, the $C_2(t)$ function was fitted to a sum of three exponentials with weights $a_i$, and decay times $\phi_i$, $i = 0, 1, 2$. The parameters for the shortest decay were obtained from a two-exponential fit to the $C_2(t)$ data for 0–25 ps, the parameters for the longest decay were obtained from a separate two-exponential fit to the $C_2(t)$ data for 200–1500 ps, and finally, the middle decay parameters were obtained by fitting the full 0–1500 ps decay with $a_0$, $\phi_0$ and $a_2$, $\phi_2$ fixed.

Statistical errors for the reported average quantities were estimated by breaking up the trajectories into 10 fragments, calculating the standard error of the mean, including the $t$ coefficient for the appropriate number of degrees of freedom at the 95% confidence level.

### Results

**Experiment: CD spectroscopy**

Analysis of temperature-dependent far-UV CD spectra was performed in order to determine the equilibrium structural and thermodynamic properties of AngI and AngII shown in Figure 1. CD spectra of AngI at both pH values (Figures 1A and 2A) have a minimum around 200 nm and decrease in ellipticity at ~230 nm as temperature increases. This is indicative of a PPII conformation (left-handed extended polyproline II) (Park et al., 1997; Pujals & Giralt, 2008; Rabanal, Ludevid, Pons, & Giralt, 1993). In a PPII conformation, the presence of an isodichroic point at ~210 nm suggests that these spectra represent a conformational transition within the random coil ensemble. The decrease in ellipticity at 230 nm suggests a change in the fractional population of the PPII conformation. Therefore as the temperature increases, the equilibrium shifts from the PPII form to an unordered conformation, causing a decrease in intensity (Park et al., 1997; Pujals & Giralt, 2008). Singular value decomposition (SVD) was applied to the combined temperature-dependent CD spectra of both samples to determine the presence of spectral components (García-Mira, Sadqi, Fischer, Sanchez-Ruiz, & Munož, 2002; Hegefeld et al., 2010; Jas & Kuczera, 2004; Konno, 1998; Sreerama, Venyaminov, & Woody, 2000). Two main components were resolved from the SVD analysis shown in Figures 1B and 2B. All other SVD components were noisy and could not be assigned to definite structural elements in a straightforward manner. The first component of AngI has a minimum at 200 nm and a maximum at 220 nm. The second component has a maximum at 200 nm. These spectra confirm that AngI exists in equilibrium between unordered and PPII conformation.

The analysis program DichroWeb (Whitmore & Wallace, 2004, 2008) was used to further analyze the structural content of AngI at both pH 4.8 and 7.2. The two reference databases that were compared included CD spectra for 22 and 17 soluble proteins in a wavelength range of 178–260 nm, respectively. These relatively small databases were chosen specifically because they contain PPII structures in their reference sets. DichroWeb analysis was averaged using CD data at 293 and 303 K in order to obtain percent structure at 298 K (Table 2). This allows for a more accurate comparison of percent structure of CD spectra with FTIR. The program provided estimates of 22% PPII, 39% unordered and 28% $\beta$-form, where $\beta$-form includes $\beta$-strand and distorted $\beta$-strand, for AngI at pH 4.8 and 21% PPII, 39% unordered, and 32% $\beta$-form for AngI at pH 7.2. The pH did not have a significant effect on AngI structural components.

Temperature dependent spectra of both AngII, pH 4.8 and 7.2 (Figures 1C and 2C), contain an isodichroic point at 208 nm, which confirms previous findings of AngII existing in a range of conformations in rapid equilibrium (Matsoukas et al., 1990). SVD analysis was also performed on AngII at both pHs and two main components were resolved (Figure 1D and Figure 2D). AngII at pH 4.8 contains a first component with a minimum at 218 nm and two maxima: at 225 nm and 240 nm. At pH 7.2, the first component for AngII has one minimum at 198 nm and two maxima at 208 nm and 225 nm. The first compo-
component of AngII at both pH's resembles that of a left-handed α-helix (Middaugh et al., 1993; Mortishire-Smith, Drake, Nutkins, & Williams, 1991). Peak positions of AngII, pH 4.8 are slightly shifted to higher wavelengths according to SVD analysis; however experimental data (Figure 2C) at low temperatures (263–283 K) contain peaks that possibly more closely resemble a left-handed helical signature. The second component of AngII, pH 4.8 has a maximum at 200 nm and a minimum at 225 nm, which is indicative of a β-form; however, the slight inflection point at 210 nm indicates the presence of a helical population. AngII at pH 4.8 may exist as a mixture of β-form and helical form causing the second component to resemble both conformations. The second component of AngII pH 7.2 has a maximum at 218 nm, and a minimum at 203 nm. There are also two slight inflections at 228 nm and 239 nm. The second component of AngII pH 7.2 is indicative of a β-turn. The presence of the two slight inflections closely resembles the signature of a helical conformation, though shifted to higher wavelengths than standard α-helical bands.

DichroWeb (Whitmore & Wallace, 2004, 2008) was also used to further analyze the structural content of AngII at both pH's. The four reference databases that were compared included CD spectra for 37, 43, 42, and 48 soluble proteins in a wavelength range of 185–240 nm. These references were chosen because of the wide variety of proteins in their databases, aimed at obtaining an accurate estimate of the conformational populations of AngII. DichroWeb analysis was averaged once again using CD data at 293 and 303 K in order to obtain percent structure at 298 K (Table 2) for more accurate comparison with FTIR data. At pH 4.8, the program calculated AngII as having 3.5% α-helix, 19% β-turn, and 31% unordered structure. AngII at pH 7.2 was found to have 3.5% α-helix, 20% β-turn, and 29% unordered structure. The remaining percentages at both pHs were calculated as being β-form. Because the references on DichroWeb do not contain left-handed structures in the databases, the percentage of β-form is considered unreliable and confirmation of the percent composition of left-handed helix could not be obtained.

As the CD spectrum of a left-handed helix could very well be a mirror image of right-handed helix, DichroWeb was used to estimate the percent composition of a possible left-handed helical population by inverting the measured angiotensin CD spectrum. Other structures could not be calculated accurately so only helical percentages were taken into account. At pH 7.2, inverted
DichroWeb analysis calculated 33% of the assumed left-handed helical population while at pH 4.8, the percentage of the possible left-handed helical population was estimated to be 28%. Inverted DichroWeb analysis thus suggests that at biological and acidic pH at 303 K, AngII contains a considerable percentage of a more compact and ordered structure, which could very well include a significant population of a left-handed helix-like conformation.

Experiment: FTIR

In order to confirm the secondary characteristics, we performed FTIR on both AngI and AngII at both pH 7.2 and pH 4.8. The amide I spectral region was analyzed to achieve a quantitative estimate of the secondary structure content with a 5% error as shown in Figure 3. Analysis of AngI pH 4.8 provided peaks centered at 1623, 1634, 1645, 1653, and 1674 cm\(^{-1}\) similar to that of AngI pH 7.2 where peaks were centered at 1625, 1636, 1645, 1654, and 1674 cm\(^{-1}\). AngII pH 4.8 deconvolution provided peaks centered at 1621, 1641, 1655, 1667, and 1698 cm\(^{-1}\) while AngII pH 7.2 had peaks centered at 1641, 1654, 1668, and 1691 cm\(^{-1}\). There were also peaks in the amide II region, which are indicative of side-chain vibrations. In all FTIR spectra, two side-chain features were evident: the peak at ~1518 cm\(^{-1}\) is characteristic of tyrosine while the peak ~1570 cm\(^{-1}\) is due to aspartic acid COO\(^{-}\) stretching (Whitmore & Wallace, 2008). Table 2 summarizes the FTIR findings for AngI and AngII.

Examination of the FTIR spectra of AngI at both pHs (Figure 3A and B) reveals the presence of a PPII helix corresponding to three peaks ~1620, 1636, and 1650 cm\(^{-1}\) in about a 3:1:1 ratio, respectively (Farrell, Wickman, Unruh, Qi, & Hoagland, 2001; Malin et al., 2001). In AngI pH 7.2, the PPII conformation encompasses 25% of its total structure, whereas AngI pH 4.8 contains 29% PPII structure. The peak around 1645 cm\(^{-1}\) is attributed to unordered structure, with 36% and 39% populations for AngI at pH 7.2 and pH 4.8, respectively. AngI at pH 7.2 also contains a peak at 1645 cm\(^{-1}\) indicating the presence of 39% \(\beta\)-form while AngI at pH 4.8 also has a peak corresponding to 32% \(\beta\)-form. As a result, FTIR confirms CD results indicating
Figure 3. FTIR of AngI and AngII (pH 7.2 and pH 4.8) deconvoluted with FSD method with a 5% error. (A) AngI pH 7.2: The blue dots indicate the raw data and the red line is the fit to the raw data. The peaks resulting from the FSD consist of a purple peak at 1625 cm\(^{-1}\), a magenta peak at 1636 cm\(^{-1}\), and a green peak at 1654 cm\(^{-1}\) indicating the presence of PPII (25%), orange peak at 1674 cm\(^{-1}\) indicating the presence of a \(\beta\)-form (39%), and cyan peak at 1645 cm\(^{-1}\) indicating the presence of unordered structure (36%). (B) AngI pH 4.8: the blue dots indicate the raw data and the red line is the fit to the raw data. The peaks resulting from the FSD consist of a green peak at 1623 cm\(^{-1}\), a magenta peak at 1634 cm\(^{-1}\), and a purple peak at 1653 cm\(^{-1}\) indicating the presence of PPII (29%), orange peak at 1674 cm\(^{-1}\) indicating the presence of a \(\beta\)-form (32%), and cyan peak at 1645 cm\(^{-1}\) indicating the presence of unordered structure (39%). (C) AngII pH 7.2: The blue dots indicate the raw data and the red line is the fit to the raw data. The peaks resulting from the FSD consist of a green peak at 1641 cm\(^{-1}\), a magenta peak at 1668 cm\(^{-1}\) indicating the presence of unordered structure (32%), cyan peak at 1668 cm\(^{-1}\) indicating the presence of a left-handed helix (43%), purple peak at 1654 cm\(^{-1}\) indicating the presence of \(\alpha\)-helix (3%), and orange peak at 1698 cm\(^{-1}\) indicating the presence of a turn (12%). (D) AngII pH 4.8: the blue dots indicate the raw data and the red line is the fit to the raw data. The peaks resulting from the FSD consist of a green peak at 1621 cm\(^{-1}\) indicating the presence of \(\beta\)-form (15%), magenta peak at 1667 cm\(^{-1}\) indicating the presence of a left-handed helix (42%), cyan peak at 1641 cm\(^{-1}\) indicating the presence of unordered structure (29%), purple peak at 1655 cm\(^{-1}\) indicating the presence of \(\alpha\)-helix (3%), and orange peak at 1698 cm\(^{-1}\) indicating the presence of a turn (12%).

Table 2. Percent secondary structure calculations obtained from the DichroWeb program and FTIR for AngI and AngII at pH 4.8 and 7.2. All results were calculated at 298 K.

|        | PPII (%) | Unordered (%) | Left-handed helix (%) | \(\alpha\)-helix (%) | \(\beta\)-turn (%) | \(\beta\)-form (%) |
|--------|----------|---------------|-----------------------|---------------------|-----------------|-----------------|
| AngI pH 7.2 | DichroWeb | 21            | 39                    | –                   | –               | 32              |
|         | FTIR     | 25            | 36                    | –                   | –               | 39              |
| AngII pH 7.2 | DichroWeb | –             | 29                    | –                   | 3.5             | 20              | 45              |
|         | FTIR     | –             | 32                    | 43                  | 3               | 22              | –               |
| AngI pH 4.8 | DichroWeb | 22            | 39                    | –                   | –               | 28              |
|         | FTIR     | 29            | 39                    | –                   | –               | 32              |
| AngII pH 4.8 | DichroWeb | –             | 31                    | –                   | 3.5             | 19              | 40              |
|         | FTIR     | –             | 29                    | 42                  | 3               | 12              | 15              |
the presence of PPII conformation in AngI at both physiological pH and in an acidic environment.

Qualitative analysis of the FTIR spectrum of AngII pH 4.8 (Figure 3D) suggests a presence of about 42% left-handed helical population as indicated by the 1667 cm\(^{-1}\) band (Elliot & Bradbury, 1962; Hashimoto & Arakawa, 1967; Siligardi & Drake, 1995). The peak at 1655 cm\(^{-1}\) indicates 3% \(\alpha\)-helical population. Peaks at 1621 and 1698 cm\(^{-1}\) are assigned to a 15% \(\beta\)-form and 12% \(\beta\)-turn, population respectively. The peak at 1641 cm\(^{-1}\) suggests the presence of a 29% unordered structure. The FTIR spectrum of AngII pH 7.2 (Figure 3C) estimated to be a 43% left-handed helical conformation represented by a band near 1668 cm\(^{-1}\) and 3% \(\alpha\)-helical conformation evidenced by a band near 1654 cm\(^{-1}\). The 1641 cm\(^{-1}\) band suggests a presence of 32% unordered structure and band near 1691 cm\(^{-1}\) can be attributed to 22% \(\beta\)-turn.

The FWHM is indicative of the distribution of secondary structures, where a wide peak will contain a greater number of secondary structural components. It is understood that AngII is a flexible peptide that is present in multiple conformations in equilibrium. The amide I peak of AngII at both pH’s have a FWHM of 62 cm\(^{-1}\), while AngI pH 4.8 and 7.2 have FWHM of 58 cm\(^{-1}\) and 55 cm\(^{-1}\), respectively, supporting the large amount of structural features found in the FSD analysis.

The percent structure calculated for the FTIR data (Table 2) of both AngI and AngII at both pH’s are consistent with the DichroWeb calculations although verification of the percentage of left-handed helix was not obtained because DichroWeb references do not contain left-handed helix reference structures. At pH 4.8, the FTIR data indicate that AngII contains a percentage of \(\beta\)-form, which is not seen at pH 7.2. At both pH 4.8 and 7.2, the majority of AngII remains as a more ordered, possibly a left-handed helical population, however, at the lower pH, His6 of AngII becomes protonated, which may result in the formation of a \(\beta\)-structure-like population. Previous studies have shown Tyr4 and His6 to be in close proximity to each other, but no hydrogen bond was detected (Spyroulias et al., 2003). Once protonated, the imidazole of His6 could interact with Tyr4, causing the structural arrangement towards a \(\beta\)-form-like population.

The FTIR and CD data clearly indicate that there exists a significant structural difference between AngI and AngII. The present structural studies indicate that the shorter AngII at both acidic and physiological pH predominantly populates a more compact structure, very possibly a left-handed helical conformation, while AngI samples PPII and unordered conformations in rapid equilibrium. PPII has often been confused with random coil because of the similarity of their CD spectra along with the lack of hydrogen bonding (Lula et al., 2007). The large percentage of PPII conformation of AngI may be the reason for AngI’s lack of biological activity.

**Experiment: fluorescence anisotropy decay measurements**

Rotational correlation times for the tyrosine residue in AngI and AngII (Table 3) were obtained by globally fitting the fluorescence decays with polarizations parallel and perpendicular to the excitation polarization as described elsewhere (Kuczera et al., 2010). The fluorescence decays and their fits are shown in Figures 4 and 5. In each case the anisotropy decay was fit with a two-exponential decay. The initial anisotropy \(r(0)\) at \(t=0\) was a variable fitting parameter.

Several trends may be observed in the data. First, the fits confirm the presence of at least two components in the anisotropy decay, a fast component \(\phi_1\) with a time constant around 50–100 ps (at 20°C), and a slow component \(\phi_2\) with a time constant of 100s of ps. The slow rotational correlation time decreases with temperature, as expected for global tumbling. In contrast, a clear temperature dependence of the fast component was not detected. The timescales of the components of the measured anisotropy decay agree semi-quantitatively with the results from MD simulations, which also show correlation times of ca. 50 ps and 300–500 ps at 300 K. The long rotational correlation time is attributable to global reorientation of the peptide. The long rotational correlation time in each case is longer for AngI than for AngII, consistent with the expectation of a smaller hydrody-

| Temperature (°C) | pH | Ang-I | Ang-II | Ang-II |
|-----------------|----|-------|--------|--------|
|                 |    | \(a_1\) | \(\phi_1\) (ns) | \(\phi_2\) (ns) | \(r(0)\) | \(\chi^2\) | \(a_1\) | \(\phi_1\) (ns) | \(\phi_2\) (ns) | \(r(0)\) | \(\chi^2\) |
| 10              | 7.2| 0.37  | 0.12   | 0.97   | 0.25  | 1.13  | 0.40  | 0.22   | 0.88   | 0.20  | 1.16  |
| 10              | 4.8| 0.40  | 0.075  | 0.82   | 0.25  | 1.22  | 0.33  | 0.12   | 0.72   | 0.22  | 1.14  |
| 20              | 7.2| 0.41  | 0.10   | 0.79   | 0.26  | 1.22  | 0.26  | 0.068  | 0.52   | 0.20  | 1.23  |
| 20              | 4.8| 0.43  | 0.053  | 0.63   | 0.24  |       | 0.26  | 0.074  | 0.48   | 0.20  | 1.20  |
| 30              | 7.2| 0.45  | 0.11   | 0.59   | 0.21  |       | 0.25  | 0.037  | 0.36   | 0.18  | 1.17  |
| 30              | 4.8| 0.42  | 0.040  | 0.45   | 0.24  | 1.10  | 0.30  | 0.087  | 0.39   | 0.18  |       |
The fast rotational correlation time $\phi_1$ corresponds to internal dynamics involving the Tyr side-chain. Comparison of the amplitudes of the two anisotropy decay components shows that the fast rotational correlation time contributes a larger fraction of the anisotropy decay for AngI than for AngII, particularly for temperatures $\geq 20^\circ$C, suggesting a greater range of internal motion for the Tyr side-chain in AngI, or, conversely, a more highly structured conformation for AngII. The lower amplitude of Tyr side-chain dynamics in AngII may reflect the presence of more side-chain contacts in AngII suggested in the MD simulations (vide infra). For AngI, the fast rotational correlation time $\phi_1$ is roughly twice as long at pH 7.2 than pH 4.8. This could indicate a more open structure at pH 4.8. There seems to be no such trend for AngII. Finally, the initial anisotropy $r(0)$ is consistently higher for AngI than for AngII. This may reveal unresolved faster motions in AngII, as suggested by the MD simulations.

**MD simulations**

The structures and dynamics explored in the four MD simulations of AngI and II are described below. Four trajectories were generated, labeled ANGIn, ANGIp, ANGIIn, and ANGIIp, with the first part of the name reflecting the simulated peptide, AngI or AngII, and the second the protonation state of the histidines, n – neutral (corresponding to normal pH) and p – protonated (lower pH).

**Conformation: structure clustering**

The results of conformational clustering for the four simulated peptides are summarized in Figure 6. In each case 50,000 structures, sampled from the MD trajectories every 40 ps were employed. For ANGIn there were 5 (Solomon & Anavekar, 2005) clusters of backbone conformations and 24 (Brooks et al., 2009) clusters for all heavy atoms, with the values denoting total cluster number (number of clusters with population above 500 members or 1% of total sampled structures). For ANGI-
In there were 2 (Lloyd-Jones et al., 2009) backbone and 13 (Yamano et al., 1995) heavy atom clusters. The results were quite similar for the protonated peptides, with 6 (Cho & Asher, 1996) backbone and 27 (Berendsen et al., 1981) heavy atom clusters for ANGlp and 3 (McManus et al., 2007) backbone and 16 (Matsoukas et al., 1990) heavy atom clusters for ANGIIp. Three trends emerge from this analysis. The first is the presence of a dominant backbone conformation, especially evident in AngII (above 90% population in top cluster), but also present in AngI (about 60% population in top cluster). The second is the large number of sampled side-chain orientations, as seen in the clustering based on heavy atom coordinates. The third is the significant decrease in flexibility of AngII relative to I. One measure of this effect is the roughly twofold decrease in the number of major clusters in the AngII trajectories compared to AngI trajectories. Plots of population history show that major clusters are visited multiple times within each of the generated trajectories, so that complete conformational sampling appears to have been achieved in our 2 μs simulations (data shown in Supplementary information).

The structures corresponding to the dominant backbone clusters are extended forms for all four simulated peptides (Figure 6, see also the discussion of the Ramachandran map below). The features present in the heavy atom clusters exhibit some differences for the different peptides. For ANGln, the top clusters generally involve few side-chain contacts; among the observed contacts were salt bridges between Arg2 and C-terminus. Other occurring structures involve: one to three central residues in turn conformation, N-terminal to C-terminal salt bridges, and contacts of several side chains (Tyr4...Phe8, Phe8...Leu10, His6...Phe8). For ANGIIln, the dominant heavy atom clusters correspond to extended structures with one or two central residues in turn conformations. There are more side-chain contacts compared to ANGln, including Tyr4 with its neighbors, Tyr4...Phe8, as well as aggregates of several side chains (e.g. residues 2,4,6,8 in cluster #2). The Arg2...C-terminal salt bridge is also present in some structures. In ANGlp the main backbone conformers are also extended, but the chain tends to be more bent than in ANGln, with larger number of residues in turn conformations. Some residues transiently adopt αR and αL conformations. The protonated histidines form

Figure 5. Fluorescence decays for AngI and AngII pH 4.8 at 10, 20, and 30 °C measured by time-correlated single-photon counting. Plots show the decay of fluorescence polarized parallel to the excitation polarization shown in blue, the decay of fluorescence polarized perpendicular to the excitation polarization shown in green, and the instrument function. Fit lines for both parallel and perpendicular fluorescence decays are shown in red. Weighted residuals are shown for the fits to the parallel (blue) and perpendicular (red) fluorescence decays. The inset illustrates the fluorescence anisotropy.
new kinds of contacts: Asp1-His6, His6-C-terminal, and His9-C-terminal. Phe8...His9 contacts also occur. For ANGIp the main conformers are extended, with presence of central residues in turn conformations. There are significant numbers of side-chain contacts, involving residues Tyr4...His6, Tyr4...Phe8, and aggregates of several side chains (Arg2...Tyr4...Phe8, Arg2...Tyr4...His6, Arg2...Tyr4...Ile5). Salt links between His6...C-terminus and Arg2...C-terminus are also present.

Conformations: Ramachandran maps
The populations of the main regions of the Ramachandran maps found in the MD trajectories are presented in Table 4. The calculation is described in Methods. The populations of the main regions of the Ramachandran map are similar when comparing ANGIp with ANGI and ANGIp with ANGIIp. Clearly, the majority of the residue backbone (\(\psi, \phi\)) conformations fall in the extended region. In all cases, the dominant conformer is PPII, followed by \(\beta\). There are also minor contributions from the right-handed and left-handed helix regions. The helical conformations are typically distorted and limited to a small number of residues, as essentially no \(\alpha\)-helical hydrogen bonds were found in the trajectory. ANGIp stands out as having lower populations of the extended structures and increased presence of helical conformations. This is the only system exhibiting a significant presence (6%) of left-handed helices. The DSSP analysis indicated a small population of 3\(_{10}\) helices for the ANGIp system. Ramachandran plots showing more details of conformations sampled in our trajectories are presented in the Supplementary information.

Conformations: secondary structure
The populations of different types of secondary structure according to the DSSP algorithm are presented in Table 5. Consistent with the previous analysis, all the peptides predominantly sample coil structures. The presence of a bend is also evident, and contributions from turn structures. Finally, a very small population of the 3\(_{10}\)-helix structure is also found, with population over 1% only for ANGIp.

Conformations: distances and contacts
Measures of peptide size and inter-residue contacts are presented in Table 6, while the distributions of the distances are shown in the Supplementary information. The radii of gyration are slightly larger for AngI compared to AngII, reflecting the presence of two more residues in the former peptide. The Ca1...Ca8 distances, measuring the extension of the part of the peptide backbone that is common in AngI and II, are quite similar in all four peptides, within the statistical errors. Overall, these results indicate a more compact structure for AngII compared to AngI, due to the truncation of the last two residues. However, the relative distances between residues 1–8 do not appear to change significantly upon angiotensin truncation. The main effect of pH change appears to be an increase in the Tyr4...Phe8 distance in the protonated peptides.

Conformations: solvent accessible surface area
We have calculated the Solvent Accessible Surface Area (SASA) of the whole peptides and the individual residues in the MD trajectories (results in Supplementary information). All residues exhibited significant SASA values during the simulations, with averages falling in the 100–200 Å\(^2\) range and fluctuations of 10–15% of the mean. There was only one residue for which the SASA exhibited statistically significant changes between the trajectories – Phe 8. For this residue, the average SASA increased from 163 Å\(^2\) for ANGI to 223 Å\(^2\) for ANGII (+60 Å\(^2\)) and from 179 Å\(^2\) for ANGIp to 226 Å\(^2\) for ANGIIp (+46 Å\(^2\)).

Conformations: hydrogen bonding
The average numbers of intramolecular hydrogen bonds found in the MD trajectories are shown in Table 7. The peptides exhibit very few hydrogen bonds, in accord with the mostly extended structures and few side-chain contacts found in the analysis above. The most definite effect seen in these results is the increase in the total number of hydrogen bonds for the peptides with protonated His compared to those with neutral His. This is in accord with the larger number of hydrogen bond donor groups present in the protonated systems.

There are several interesting effects of the protonation of the His residues seen in the MD trajectories. In
terms of sampled structures, the protonated His residues tend to form salt bridges with either Asp1 or the C-terminus, the backbone tends to sample more structures in the helical range – mostly right-handed, but also some left-handed helices (especially for ANGIp). The protonated forms also tend to form a larger number of intramolecular hydrogen bonds. All these effects are stronger when comparing ANGIp with ANGIn, and weaker for the ANGIIp/ANGIIn pair.

**Dynamics: reorientation timescales**

To estimate the correlation times involved in the overall peptide reorientations, we used the autocorrelation function of the end-to-end vector (Table 8 and Supplementary
Table 4. Percentage populations of the main regions of the Ramachandran map from MD simulations. Details explained in Methods.

|       | PPII  | β      | α_R   | α_L  |
|-------|-------|--------|-------|------|
| ANGln | 61 ± 4| 42 ± 3 | 14 ± 3| 1 ± 1|
| ANGIln| 68 ± 5| 44 ± 4 | 13 ± 3| 0    |
| ANGIp | 48 ± 6| 34 ± 5 | 18 ± 5| 6 ± 3|
| ANGIlp| 62 ± 6| 42 ± 5 | 16 ± 5| 1 ± 1|
The timescales were obtained by fitting the second-order autocorrelation functions of the corresponding axis vectors. The end-to-end vector reorientation decays may be represented by three correlation times. These decays exhibited a short component of about 1 ps, an intermediate component of 60–70 ps, and a long component of 400–500 ps. The short decay may be assigned to vibrational relaxation, the intermediate to global conformational dynamics, and the long one to overall peptide tumbling. The conformational relaxation is slightly slower in the AngI simulations (69–70 ps) compared to AngII (55–59 ps). The peptide tumbling is also slower in the longer peptides (460–470 ps) compared to the shorter ones (370 ps). This last trend is consistent with the smaller radius of gyration found for AngII.

The reorientation times for the transition dipole axis of Tyr4 determined from the MD simulations are also presented in Table 8. The shortest time, 1.5 ps for all peptides, may be assigned to vibrational motions. The intermediate time, about 60 ps in AngI and about 50 ps in Ang, represents Tyr side-chain reorientations due to local conformational dynamics. The slowest timescales are about 400 ps in AngI and about 300 ps in AngII; these times are usually assigned to overall peptide tumbling.

These two shorter relaxation times of the transition dipole may be compared to the two timescales, 2.0 and 22 ps, found in our control 50 ns simulation of the blocked Tyr peptide, solvated by 864 SPC waters, with OPLS-AA force field. The faster timescales are in good agreement, while the slower times are 2–3 times longer in the angiotensins, indicating that local side-chain motions are hindered in our peptides compared to blocked Tyr itself. Comparing with the end-to-end distance results, the local Tyr relaxation is somewhat faster than the global conformational dynamics (56–59 ps vs. 69–70 ps for AngI and 46–50 vs. 55–59 ps for AngII). Similarly, the slowest relaxation of Tyr4 is systematically faster than the overall tumbling as seen in the end-to-end distance reorientation (380 vs. 460–470 ps for AngI and 300–310 ps vs. 370 ps for AngII). Thus, the local dynamics reported by Tyr4 reorientations is similar, but not identical to the global peptide dynamics as seen in the end-to-end-distance.

Table 5. Percentage populations of secondary structure in the MD trajectories based on DSSP analysis.

|         | Coil | Bend | Turn | 3_{10} |
|---------|------|------|------|--------|
| ANGI_n  | 80   | 17   | 3    | 0      |
| ANGI_{ln}| 80   | 17   | 3    | 0      |
| ANGI_{lp}| 82   | 15   | 1    | 0      |
| ANGI_{lp}| 82   | 10   | 5    | 2      |

Table 6. Measures of average peptide size from the MD trajectories. Distances are in Å. $R_g$ is the radius of gyration calculated for all protein atoms. The distance between $\text{Ca}$ atoms of residues 1 and 8 is used to compare the end-to-end separation. Distances between side-chain centers of mass compare side-chain separations. Units: Å.

|         | $R_g$ | $\text{Ca1-Ca8}$ | Tyr4…Arg2 | Tyr4…His6 | Tyr4…Phe8 |
|---------|-------|------------------|-----------|-----------|-----------|
| ANGI_n  | 7.8±0.3 | 16.0±0.8        | 8.1±0.8  | 9.0±0.5  | 11.0±1.4 |
| ANGI_{ln}| 6.9±0.3 | 15.7±0.8        | 7.4±0.8  | 7.6±0.5  | 11.5±1.1 |
| ANGI_{lp}| 7.6±0.4 | 15.3±1.4        | 7.8±0.4  | 8.7±0.4  | 13.8±1.5 |
| ANGI_{lp}| 6.9±0.4 | 15.2±1.2        | 7.2±0.5  | 7.9±0.5  | 12.2±0.5 |

Table 7. Average number of hydrogen bonds found in the MD trajectories. Total – for all pairs of atoms; main chain – involving only main-chain atoms.

|         | Total   | Main chain |
|---------|---------|------------|
| ANGI_n  | 1.6±0.2 | 0.3±0.1    |
| ANGI_{lp}| 2.9±0.3 | 0.9±0.2    |
| ANGI_{ln}| 1.0±0.1 | 0.12±0.04  |
| ANGI_{lp}| 1.4±0.2 | 0.4±0.1    |

Table 8. Decay times (in ps) and amplitudes in the decays of $C_2(t)$ autocorrelation functions of the transition dipole and end-to-end vector from MD simulations. The statistical error estimates are about 3% for the longest timescales and less for the shorter ones.

|         | Transition dipole | End-to-end vector |
|---------|------------------|------------------|
|         | $a_1$ | $\phi_1$ | $a_2$ | $\phi_2$ | $a_3$ | $\phi_3$ | $a_1$ | $\phi_1$ | $a_2$ | $\phi_2$ | $a_3$ | $\phi_3$ |
| ANGI_n  | 0.18  | 1.5   | 0.26  | 59    | 0.56  | 380   | 0.06  | 1.4   | 0.29  | 70    | 0.65  | 460   |
| ANGI_{lp}| 0.17  | 1.5   | 0.25  | 56    | 0.58  | 380   | 0.03  | 1.3   | 0.18  | 69    | 0.79  | 470   |
| ANGI_{ln}| 0.18  | 1.5   | 0.22  | 46    | 0.60  | 300   | 0.06  | 1.4   | 0.28  | 59    | 0.66  | 370   |
| ANGI_{lp}| 0.17  | 1.5   | 0.23  | 50    | 0.60  | 310   | 0.06  | 1.4   | 0.27  | 55    | 0.67  | 370   |
The decays presented in Table 8 show that the effect of His protonation on the peptide reorientations is small, both at the global and local level. The effect of truncation of the last two residues is significant, leading to faster conformational relaxation and faster overall tumbling.

Comparison with experimental data

Tables 9 and 10 show a summary of the comparison of our trajectories with published experimental structures of angiotensin (Spyroulias et al., 2003). AngI and AngII structures that are within 1 Å in backbone RMSD and within 3 Å non-H atom RMSD of the experimental conformers may be found in our MD trajectories. For comparison, we show statistics of differences between the trajectory ensembles and selected simulation structures — the starting structure (extended form) and the central structure from the top non-H atom cluster. The statistics for experimental–trajectory and trajectory–trajectory RMSD are quite similar, suggesting that we can consider the experimental structures to be part of our MD ensemble (Garcia, Ronco, Brunger, & Amzel, 1992; Lula et al., 2007). Overall, this analysis indicates a good overlap between the ensemble of structures sampled in our trajectories and the known experimental structures of angiotensin.

Discussion

Structural characterization of AngI and AngII has been carried out by several spectroscopic techniques such as far-UV CD spectroscopy, NMR spectroscopy, and FTIR spectroscopy. Previously, results have shown AngI to exist mainly in a disordered state with an increase in β-sheet population above pH 7.0 (Spyroulias et al., 2003; Venkateshwaran, Stewart, Bishop, de Haseth, & Bartlett, 1998). Small linear peptides usually exist in solution as a range of conformations in rapid equilibrium (Matsumukas et al., 1990). Accordingly, interpretation of the conformational studies of AngII are conflicting, which has led to various structural models in the solution including β-pleated sheet, β and γ turns, and other structures (Carpenter, Wilkes, & Schiller, 1998; Greff et al., 1976; Lintner et al., 1977; Spyroulias et al., 2003; Tzakos et al., 2004; Vieira et al., 2009). One reason for contradicting results could possibly be due to solvent effects. For example, the measurements of Litner et al. were performed in TFE, which is a known helix inducer, and may induce the formation of helices, which may otherwise be absent in the native environment. This could explain the observation of a right-handed α-helix for AngII and its analogs by those authors. Vieira et al. looked at native AngII and TOAC labeled AngII at pH 4, 7, and 10 at room temperature, which could cause

| Structure | Ca RMSD | Non-H atom RMSD |
|-----------|---------|-----------------|
|           | Mean ± S.D. | Min. | Max. | Mean ± S.D. | Min. | Max. |
| 2JP8      | 2.2 ± 0.4   | 0.8   | 3.8   | 4.2 ± 0.7   | 2.0  | 5.8   |
| 3CK0      | 2.6 ± 0.7   | 0.4   | 5.3   | 4.9 ± 0.8   | 2.3  | 7.6   |
| 1N9U      | 3.4 ± 0.7   | 0.9   | 6.3   | 5.2 ± 0.7   | 2.5  | 7.6   |
| Start     | 3.9 ± 1.4   | 0.6   | 8.2   | 5.6 ± 1.2   | 1.8  | 9.3   |
| Top       | 4.0 ± 1.6   | 0.0   | 8.5   | 5.7 ± 1.5   | 0.0  | 9.5   |

Table 10. RMSD of selected experimental structures from ANGIn MD trajectory. The data presented are the statistics for RMSD values over 2,000,000 structures sampled every 1 ps: mean, standard deviation of the mean (95% confidence interval), minimum and maximum. Units: Å. Structures as in Table 9. Only residues 1–7 of ANGII overlaid on 2JP8 and residues 1–8 were overlaid on ANGIn.
changes to the native structure. In addition, Figure 6, top row, of Vieira et al. presents very noticeable similarities with our CD scans, as do the Litner et al. CD scans of native AngII in pH 5 (close to the data in this manuscript, pH 4.8) in their Figure 3. In order to clearly understand the structure and dynamics of the biologically interesting AngI and AngII peptides, we have carried out a joint experimental and computational study of the structure and reorientation dynamics.

**Summary of experimental results**

In the current study, clear structural differences between AngI and AngII have been established by far-UV CD spectroscopy, FTIR spectroscopy, and rotational anisotropy. The most important structural feature is that the active form of angiotensin (AngII) is observed to have a significantly more compact and ordered conformation with the possible existence of a left-handed helical population, while AngI exists as unordered and PPII conformations in equilibrium. The lack of defined structure in AngI may explain the inactivity of AngI. At acidic pH, AngII also contains a population of 15% β-form. Even though AngI contains two His residues and AngII contains one His, changes from acidic to neutral pH were found to have very little effect on their conformational populations.

The presence of unordered conformations and β-form is in agreement with FTIR results from Venkateshwaran et al. (1998). PPII has often been confused with random coil because of the similarity of their CD spectra along with the lack of hydrogen bonding (Siligardi & Drake, 1995; Tiffany & Krimm, 1968, 1972). For these reasons, it is possible that some results for AngI were incorrectly assigned to disordered population. There appears to be no consensus on the multiple conformations of AngII. Our CD and FTIR results determined the main population of AngII to be a left-handed helix, which is in agreement with Kataoka, Beusen, Clark, Yodo, and Marshall (1992).

Anisotropy measurements were performed in order to gain insight into the dynamic behavior of AngI and AngII. The local and global orientation dynamics observed with anisotropy of AngI and AngII support the measured structural differences. The amplitude indicates that the Tyr4 side-chain is in a more rigid, restrictive environment in AngII compared to AngI. This is an indication that AngII exhibits more compact conformational packing compared to AngI. The long rotational correlation time in each case is longer for AngI than for AngII, which is consistent with expectations, since AngI and AngII have ten and eight residues, respectively.

**Summary of MD results**

Several effects of truncation of the last two residues of AngI may be found in the MD simulations. First, the truncated peptide becomes less flexible, with the number of sampled conformations decreasing by about a factor of 2 compared to the full system. Both AngI and II systems populate mostly extended structures with the side chains extending into the solvent. The shorter peptides tend to be more compact, with shorter radii of gyration, fewer intramolecular hydrogen bonds, but with comparable side chain–side chain packing as the longer forms. The backbone tends to populate extended forms from the PPII and β families, with some presence of turns and minor contributions from right- and left-handed helical states. The side chains are mostly extended towards the solvent, with few contacts on average. The simulations predict very little change in angiotensin structure upon lowering of pH, aside from an increase in intramolecular hydrogen bonding involving the protonated histidine(s). The predicted structural differences between the AngI precursor and AngII are subtle, but well-defined. The MD results show quite similar backbone conformations for both peptides. The novel structural features that appear in AngII are: presence of Arg2–C-terminal salt bridges, a more compact structure, tighter packing of side chains and marked increase in solvent exposure of Phe8 relative to AngI.

A significant structural feature is the increase in solvent accessibility of Phe8 in AngII compared to AngI. This might be expected for a terminal residue in a short peptide, but could play an important role in increasing the affinity of AngII for a hydrophobic receptor site. Thus, the angiotensin 1–7 peptide, corresponding to AngII with Phe8 deleted, exhibits activity counteractive to AngII itself (Ferrario, 1998). In terms of dynamics, the main effect is the decrease of the overall tumbling time upon peptide truncation, consistent with the decrease of the radius of gyration.

**Comparison of experimental and MD results**

The simulation results mostly agree with the experimental data on the details of angiotensin structure and dynamics presented in this work. The MD agrees with the far-UV CD and FTIR results that the coil and turn secondary structures are important components for all four simulated angiotensin systems. The MD results predict that the main backbone conformer populations should fall in the region of extended forms – PPII and β. There are some discrepancies between simulations and CD/FTIR, which may partly be explained by the different classification schemes employed. Due to the large size of the basins used in the MD conformational analysis, some of the trajectory structures classified as β, PPII, and αR will appear as “coil” in the experimental data. The largest discrepancy involves the appearance of left-handed helix conformers seen for AngII in far-UV CD and FTIR. These results are not reproduced by the MD simulations, which generally show only quite low contributions of 1–6% of left-handed helices, with no systematic increase found between AngI and II trajectories.
Generally, the experimental data show large structural changes upon peptide truncation and small changes with pH. For the MD simulation results, there are relatively subtle changes with truncation and very little change with pH. The anisotropy decay results from experiments and simulations are also in good qualitative agreement, identifying conformational relaxation on the timescale of several tens of picoseconds and an overall reorientation on the timescale of several hundreds of picoseconds. The faster overall tumbling time for AngII compared to AngI is correctly reproduced by the MD, and is consistent with the calculated radii of gyration. The experimentally observed effect of lower amplitude of the local dynamics component in AngII compared to AngI is also found in the MD results, although it is less pronounced. The simulation predictions for the global reorientation are systematically faster than the measured values. This is consistent with the underestimation of the viscosity of water by simple water models (Mark & Nilsson, 2001). The finer details of the experimental data – the markedly slower conformational relaxation of ANGInp compared to ANGIp and the slower tumbling of ANGIn compared to ANGIp – are not detected in the MD trajectories.

The computational results mostly agree with experimental data. There is a good overlap between the ensemble of trajectory structures and available experimental structures of the angiotensins. The sampled conformations are also mostly in accord with CD and FTIR results presented in this work. The primary discrepancy is in the importance of left-handed helices, which accounts for about 30% of the conformations of AngII in the experimental measurements, but is found only at 1–6% levels in the trajectories. In our view, the experimental observations should be given greater weight than the MD simulations. Protein force field parameterization is not a perfect procedure, typically based on quantum chemical calculations of model systems in vacuum or on condensed phase experimental observations for large peptides/proteins with well-ordered three-dimensional structures. Thus, some discrepancies between observation and modeling may occur for small, flexible peptides in solution.

The good overall agreement of calculations with experimental data allows us to attempt to draw conclusions about functional consequences from the detailed microscopic results of MD. Several features found in the simulations may be correlated with previously proposed explanations of differential interactions between AngI and AngII with the angiotensin receptors (Spyroulias et al., 2003). The smaller size of AngII found in simulations and anisotropy measurements may explain its selective binding to AT1 and AT2. The twisted-extended AngII active structure proposed by Marshall and coworkers is similar to the NMR-determined models (Preto, Melo, Maia, Mavromoustakos, & Ramos, 2005) as well as to several clusters sampled in MD. The extended and U-shaped structures proposed for AngII/receptor complexes may also be found in our trajectories. The increased solvent exposure of Phe8 seen in AngII MD may be correlated with the proposed role of Phe8 and its exposed carboxylate group in AngII/receptor binding (Spyroulias et al., 2003).

**Ordered and compact AngII population in solution**

Our experimental observations provide support for the presence of a subset of population that may be consistent with a \( \alpha \)-helical conformation in AngII. In the AngII CD spectra at low temperatures, the maxima and minimum resemble the mirror image of the signature signal of the right-handed helical conformation. Our native AngII CD spectrum contains a maximum at 220\( \text{nm} \) which is assigned to be a characteristic marker band for the presence of a left-handed helical conformation. The appearance of this band may not be due solely to the presence of Phe and Tyr in the peptide sequence. Phe and Tyr produce bands at 218\( \text{nm} \) and 228\( \text{nm} \), respectively, in the CD spectrum. However, the far-UV CD spectra of these aromatic amino acids do not exhibit changes in the measured ellipticity as a function of temperature, while the observed CD spectral bands of AngII at 220 and 200\( \text{nm} \) do show such changes.

**Conclusions**

In this study a clear structural difference between AngI and AngII has emerged. Previous studies have demonstrated AngI and II existing as very dynamic molecules with highly flexible termini in aqueous solution (Spyroulias et al., 2003; Zhang et al., 1996). The MD simulation results also suggest that the peptides are very flexible, sampling a large number of conformations, which differ primarily in side-chain orientations. The wide range of structures includes ones that are similar to those previously considered as biologically active (Noda, Saad, & Karnik, 1995; Spyroulias et al., 2003).

Even though AngI contains all eight amino acids of AngII, it has no known biological activity and acts only as a precursor to AngII, a potent vasoconstrictor. Experimentally, we have found AngI exists in equilibrium between unordered conformations and a PPII helix along with a subpopulation of \( \beta \)-form. Subpopulations were found to consist of \( \beta \)-turn, unordered, and a small population of \( \alpha \)-helix conformations. The lack of defined structure in AngI could explain why it has no known biological function. The lack of activity could be due to the majority of “unordered” structure (PPII and unordered conformation) in AngI.

The most intriguing structural feature observed here is the population of more compact conformations by the shorter, biologically active AngII peptide. These structures could possibly include a mirror image of the right-handed helix. In contrast AngI exists in equilibrium between unordered conformation and the PPII helix. Previous studies have shown the binding site of AngII is located at the membrane–water interface. Based on the
structural analysis and orientation dynamics in this study, we suggest that the existence of a significant population of more ordered and compact, possibly left-handed helical conformation in AngII at both physiological and acidic pH, could present the necessary and critically important structural component for receptor binding and activation. It is also interesting to note that angiotensin 1–7 (Ang₁₋₇), which functions as an antagonist to AngII, has a very distinct minimum at 220 nm in the far-UV CD spectrum (De Leon and Jas, xxxx), indicating a significant population of right-handed α-helical conformation.

In conclusion, the combined experimental and MD approach presented here suggests that truncation of AngI to form AngII introduces restrictions in the populations of the available range of structures that enable biological activity in AngII. These features include a more compact average structure with more restricted internal dynamics as well as greater exposure of the terminal Phe.

Acknowledgments

We would like to acknowledge the Baylor University Academic and Research Computer Services for use of computer resources and Mike Hutchenson for technical assistance in the computational part of the project. This project was supported in part by URG Baylor funding for GSJ and Big XII summer fellowships from the University of Kansas for KK.

Supplementary material

The supplementary material for this paper is available online at http://dx.doi.org/10.1080/07391102.2011.672631.

References

Aplin, M., Christensen, G.L., Schneider, M., Heydorn, A., Gammeltoft, S., Kjølbye, A.L., … Hansen, J.L. (2007). Differential extracellular signal-regulated kinases 1 and 2 activation by the angiotensin Type 1 receptor supports distinct phenotypes of cardiac myocytes. Basic & Clinical Pharmacology & Toxicology, 100, 296–301.

Berendsen, H.J.C., Postma, J.P.M., van Gunsteren, W.F., & Hermans, J. (1981). Interaction models for water in relation to protein hydration. Journal of Intermolecular Forces, 331–342.

Boucard, A.A., Wilkes, B.C., Laporte, S.A., Escher, E., Guilletmette, G., & Leduc, R. (2000). Photolabeling identifies position 172 of the human AT1 receptor as a ligand contact point: Receptor-bound angiotensin II adopts an extended structure. Biochemistry, 39, 9662–9670.

Brooks, B.R., Brooks, C.L.III, MacKerell, A.D.Jr., Nilsson, L., Petrella, R.J., Roux, B., … Karplus, M. (2009). CHARMM: The biomolecular simulation program. Journal of Computational Chemistry, 30, 1545–1615.

Buchete, N.V., & Hummer, G. (2008). Peptide folding kinetics from replica exchange molecular dynamics. Journal of Physical Chemistry B, 112, 6057–6069.

Carpenter, K.A., Wilkes, B.C., & Schiller, P.W. (1998). The octapeptide angiotensin II adopts well-defined structure in a phospholipid environment. European Journal of Biochemistry, 251, 448–453.

Chilman-Blair, K., & Rabuseda, X. (2003). Olmesartan, an AT1-selective antihypertensive agent. Drugs Today, 39, 745–761.

Cho, N., & Asher, S.A. (1996). UV resonance Raman and absorption studies of angiotensin II conformation in lipid environments. Biospec, 2, 71–82.

Daura, X., Gademann, K., Jaun, B., & Mark, A.E. (1999). Peptide folding: When simulation meets experiment. Angewandte Chemie-International, 38, 236–240.

Daura, X., Gademann, K., Schafer, H., Jaun, B., Seebach, D., & van Gunsteren, W.F. (2001). The β-peptide hairpin in solution: Conformational study of a β-hexapeptide in methanol by NMR spectroscopy and MD simulation. Journal of the American Chemical Society, 123, 2393–2404.

De Leon, K., & Jas, G.S. Unpublished results.

Deraet, M., Rihakova, L., Boucard, A., Perodin, J., Sauer, S., Mathieu, A., … Escher, E. (2002). Angiotensin II is bound to both receptors AT(1) and AT(2), parallel to the transmembrane domains and in an extended form. Canadian Journal of Physiology and Pharmacology, 80, 418–425.

Elliot, A., & Bradbury, E.M. (1962). Journal of Molecular Biology, 5, 574–576.

Farrell, H.M., Wickman, E.D., Unruh, J.J., Qi, P.X., & Hoagland, P.D. (2001). Food Hydrocolloids, 15, 341–354.

Fernández, C.M. (1998). Angiotensin-(1–7) and antihypertensive mechanisms. Journal of Nephrology, 11, 278–283.

Garcia, K.C., Desiderio, S.V., Ronco, P.M., Verroust, P.J., & Amzel, L.M. (1992). Recognition of angiotensin II: Antibodies at different levels of an idiotypic network are superimposable. Science, 257, 528–531.

Garcia, K.C., Ronco, P.M., Brunger, A.T., & Amzel, L.M. (1992). Three-dimensional structure of an angiotensin II-Fab complex at 3 Å: Hormone recognition by an anti-idiotypic antibody. Science, 257, 502–507.

Garciá-Mira, M.M., Sadqi, M., Fischer, N., Sanchez-Ruiz, J.M., & Munoz, V. (2002). Experimental identification of downhill protein folding. Science, 298, 2191–2195.

Greff, D., Fermandjian, S., Fromageot, P., Khosla, M.C., Smeby, R.R., & Bumpus, F.M. (1976). Circular dichroism spectra of truncated and other analogs of angiotensin II. European Journal of Biochemistry, 61, 297–305.

Hashimoto, M., & Arakawa, S. (1967). Studies of Poly-β-benzyl-L-aspartate Helix. III. Infrared Spectra of Copolymers of β-Benzyl-L-aspartate with β-Methyl Chloro, Cyano, or Nitrobenzyl-L-aspartate in a Chloroform Solution. Bulletin of the Chemical Society of Japan, 40, 1698–1701.

Hegefeld, W.A., Chen, S., DeLeon, K.Y., Kuczera, K., & Jas, G.S. (2010). Helix formation in a pentapeptide: Experiment and force-field dependent dynamics. Journal of Physical Chemistry A, 114, 12391–12402.

Hess, B., Kutzner, C., van der Spoel, D., & Lindahl, E. (2008). Algorithms for highly efficient, load-balanced, and scalable molecular simulation. Journal of Chemical Theory and Computation, 4, 435–447.

Jas, G.S., & Kuczera, K. (2004). Equilibrium structure and folding of a Helix-Forming peptide: Circular dichroism measurements and replica-exchange molecular dynamics simulations. Biophysical Journal, 87, 3786–3798.

Kabsch, W., & Sander, C. (1983). Dictionary of protein secondary structure: Pattern recognition of hydrogen-bonded and geometrical features. Biopolymers, 22, 2577–2673.

Kaminki, G.A., Friesner, R.A., Tirado-Rives, J., & Jorgensen, W.L. (2001). Evaluation and reparametrization of the OPLS-AA force field for proteins via comparison with accurate quantum chemical calculations on peptides. Journal of Physical Chemistry B, 105, 6474–6487.

Katoaka, T., Beussen, D.D., Clark, J.D., Yodo, M., & Marshall, G.R. (1992). The utility of side-chain cyclization in determining the receptor-bound conformation of peptides - cyclic tripeptides and angiotensin-II. Biopolymers, 32, 1519–1533.
Konno, T. (1998). Conformational diversity of acid-denatured cytochrome c studied by a matrix analysis of far-UV CD spectra. *Protein Science, 7*, 975–982.

Kuczera, K., Unruh, J., Johnson, C.K., & Jas, G.S. (2010). Reorientation of aromatic amino acids and their side chain models: Anisotropy measurement and molecular dynamics simulations. *Journal of Physical Chemistry A, 114*, 133–142.

Lintner, K., Fernandijian, S., Fromageot, P., Khosla, M.C., Smeyth, R.R., & Bumpus, F.M. (1977). Circular dichroism studies of angiotensin II and analogues: Effects of primary sequence, solvent, and pH on the side chain conformation. *Biochemistry, 16*, 806–814.

Lloyd-Jones, D., Adams, R., & Carnethon, M. (2009). Heart disease and stroke statistics—2009 update: A report from the American heart association statistics committee and stroke statistics Subcommittee. *Circulation, 119*, e21–e181.

Lula, I., Denadai, A.L., Resende, J.M., de Sousa, F.B., de Lima, G.F., Pilo-Veroso, D., … Sinisterra, R.D. (2007). Study of angiotensin-(1-7) vasoactive peptide and its -cyclodextrin inclusion complexes: Complete sequence-specific assignments and structural studies. *Peptides, 28*, 2199–2212.

Malin, E.L., Alaimo, M.H., Brown, E.M., Aramini, J.M., Germann, M.W., Farrell, H.M.I., … Fox, P.F. (2001). Solution Structures of Casein Peptides: NMR, FTIR, CD, and Molecular Modeling Studies of u1-Casein, 1-23. *Journal of Protein Chemistry, 20*, 391–404.

Mark, P., & Nilsson, L. (2001). Structure and dynamics of the TIP3P, SPC and SPC/E water models at 298 K. *Journal of Physical Chemistry A, 105*, 9954–9960.

Matsoukas, J.M., Bigam, G., Zhou, N., & Moore, G.J. (1990). 1H-NMR studies of [Sar1]angiotensin II conformation by nuclear overhauser effect spectroscopy in the rotating frame (ROESY): Clustering of the aromatic rings in dimethylsulfoxide. *Peptides, 11*, 359–366.

McManus, F., Freel, E.M., & Connell, J.M.C. (2007). Hypertension. *Scottish Medical Journal, 52*, 36–42.

Middaugh, C.R., Thomson, J.A., Burke, C.J., Mach, H., Naylor, A.M., Bogusky, M.J., … Thomson, J.A. (1993). Structure of synthetic peptide analogues of an egg shell protein of Schistosoma mansoni. *Protein Science, 2*, 900–914.

Mortishire-Smith, R.J., Drake, A.F., Nutkins, J.C., & Williams, D.H. (1991). Left-handed u-helix formation by a bacterial peptide. *FEBS Letters, 278*, 244–246.

Noda, K., Saad, Y., & Karnik, S.S. (1995). Interaction of Phe of Angiotensin II with Lys and His of AT1 Receptor in Agonist Activation. *Journal of Biological Chemistry, 270*, 28511–28514.

Oliveira, L., Costa-Neto, C.M., Nakaie, C.R., Schreier, S., Shimuta, S.I., & Paiva, A.C.M. (2007). The Angiotensin II AT1 receptor structure-activity correlations in the light of rhodopsin structure. *Physiological Reviews, 87*, 565–592.

Park, S., Shalongo, W., & Stellwagen, E. (1997). The role of PII conformations in the calculation of peptide fractional helix content. *Protein Science, 6*, 1694–1700.

Piana, S., Lindorf-F Larsen, K., & Shaw, D.E. (2011). How robust are protein folding simulations with respect to force field parameterization? *Biophysical Journal, 100*, L47–L49.

Pretto, M.A.C., Melo, A., Maia, H.L.S., Mavromoustakos, T., & Ramos, M.J. (2005). Molecular dynamics simulations of angiotensin II in aqueous and dimethyl sulfoxide environments. *Journal of Physical Chemistry B, 109*, 17743–17751.

Pujals, S., & Giralt, E. (2008). Proline-rich, amphipathic cell-penetrating peptides. *Advanced Drug Delivery Reviews, 60*, 473–484.

Puska, P., Mendis, S., & Porter, D. (2003). World health organization chronic disease risk factors: Key risk factors include high cholesterol, high blood pressure, low fruit and vegetable intake. *World Health Organization*.

Rabanal, F., Ludevid, M.D., Pons, M., & Giralt, E. (1993). CD of proline-rich polypeptides - application to the study of the repetitive domain of maize glutelin-2. *Biopolymers, 33*, 1019–1028.

Sagui, C., & Darden, T.A. (1999). Molecular dynamics simulations of biomolecules: Long-range electrostatic effects. *Annual Review of Biophysics and Biomolecular Structure, 28*, 155–179.

Siligardi, G., & Drake, A.F. (1995). The importance of extended conformations and, in particular, the PII conformation for the molecular recognition of peptides. *Biopolymers, 37*, 281–292.

Sköld, C., Nikiforovich, G., & Karlén, A. (2008). Modeling binding modes of angiotensin II and pseudopeptide analogues to the AT2 receptor. *Journal of Molecular Graphics and Modelling, 26*, 991–1003.

Solomon, S.D., & Anavekar, N. (2005). A brief overview of inhibition of the Renin-Angiotensin system: Emphasis on blockade of the Angiotensin II Type-1 Receptor. *Medscape Cardiology, 9(2)*, 1–6.

Sorin, E.J., & Pande, V.S. (2005). Exploring the Helix-Coil transition via all-atom equilibrium ensemble simulations. *Biophysical Journal, 88*, 2472–2493.

Spyroulias, G.A., Nikolakopoulou, P., Tzakos, A., Gerothanassis, I.P., Magafa, V., Manessi-Zoupa, E., & Cordopatis, P. (2003). Comparison of the solution structures of angiotensin I & II Implication for structure-function relationship. *European Journal of Biochemistry, 270*, 2163–2173.

Srerama, N., Venyaminov, S.Y., & Woody, R.W. (2000). Estimation of protein secondary structure from circular dichroism spectra: Inclusion of denatured proteins with native proteins in the analysis. *Analytical Biochemistry, 287*, 243–251.

Tiffany, M.L., & Krimm, S. (1968). Communications to the editor: New chain conformations of poly(glutamic acid) and polylysine. *Biopolymers, 6*, 1379–1382.

Tiffany, M.L., & Krimm, S. (1972). Effect of temperature on the circular dichroism spectra of polypeptides in the extended state. *Biopolymers, 11*, 2309–2316.

Tzakos, A.G., Gerothanassis, I.P., & Troganis, A.N. (2004). On the Structural Basis of the Hypertensive Properties of Angiotensin II: A Solved Mystery or a Controversial Issue? *Current Topics in Medicinal Chemistry, 4*, 431–444.

Unruh, J.R. (2006). Development of fluorescence spectroscopy tools for the measurement of biomolecular dynamics and heterogeneity. Ph.D. Thesis, University of Kansas, Lawrence, Kansas, p. 237.

Venkateshwaran, T.G., Stewart, J.T., Bishop, R.T., de Haseth, J.A., & Bartlett, M.G. (1998). Solution conformation of model peptides with the use of particle beam LC/FT-IR spectrometry and electrospray mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis, 17*, 57–67.

Vieira, R.F.F., Casallanovo, F., Marin, N., Paiva, A.C.M., Schreier, S., & Nakaie, C.R. (2009). Conformational properties of angiotensin II and its active and inactive TOAC-labeled analogs in the presence of micelles. Electron paramagnetic resonance, fluorescence, and circular dichroism studies. *Biopolymers, 92*, 525–537.

Whitmore, L., & Wallace, B.A. (2004). DICHRoWEB: An online server for protein secondary structure analyses from circular dichroism spectroscopic data. *Nucleic Acids Research, 32*, W668–W673.
Whitmore, L., & Wallace, B.A. (2008). Protein secondary structure analyses from circular dichroism spectroscopy: Methods and reference databases. Biopolymers, 89, 392–400.

Yamano, Y., Ohyama, K., Kikyo, M., Sano, T., Nakagomi, Y., Inoue, Y., ... Inagami, T. (1995). Mutagenesis and the molecular Modeling of the Rat Angiotensin II Receptor (AT1). Journal of Biological Chemistry, 270, 14024–14030.

Yeh, I.C., & Hummer, G. (2002). Peptide loop-closure kinetics from microsecond molecular dynamics simulations in explicit solvent. Journal of the American Chemical Society, 124, 6563–6568.

Zhang, W.-J., Nikiforovich, G.V., Perodin, J., Richard, D.E., Escher, E., & Marshall, G.R. (1996). Novel cyclic analogs of angiotensin II with cyclization between positions 5 and 7: Conformational and biological applications. Journal of Medicinal Chemistry, 39, 2738–2744.