Interactions of Trifluoroethanol with [val^5]angiotensin II

Chiradip Chatterjee, David Martinez, and John T. Gerig*

Department of Chemistry and Biochemistry, University of California, Santa Barbara, Santa Barbara, California 93106

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Intermolecular $^1H\{^{19}F\}$ NOE experiments have been used to explore the interactions of trifluoroethanol (TFE) with the octapeptide hormone [val^5]angiotensin II at temperatures from 5 to 25 °C. Circular dichroism spectra indicate that 40% trifluoroethanol has an influence on the conformations of the peptide, probably leading to β-structures. Diffusion experiments show that the mean hydrodynamic radius of the peptide in 40% trifluoroethanol−water is about 8 Å, consistent with significant folding of the peptide in this medium. Distance constraints derived from intramolecular NOESY data along with observed vicinal coupling constants ($^3J_{C\alpha HNH}$) were used to develop conformations consistent with available data. Assuming that intermolecular $^1H\{^{19}F\}$ NOEs are the result of diffusive encounters of TFE and peptide molecules, it is shown that no single conformation is consistent with the experimental values of the σ_{HF} cross-relaxation parameters. It is argued that the disagreements between observed and expected values of σ_{HF} are the result of formation of long-lived (∼0.5 ns) fluoroalcohol−peptide complexes, a conclusion consonant with similar studies of other peptide−fluoroalcohol systems. Complex formation appears to be especially prevalent near the charged amino acid side chains of the hormone.

Introduction

The octapeptide hormone angiotensin II is a key component of the renin−aldosterone−angiotensin system in humans and other species and plays a central role in the regulation of blood pressure and blood volume. The three-dimensional structure of the hormone, especially as it interacts with its receptors, has been of continuing interest in the context of drug development.[1−3] While it is anticipated that a small peptide may not have a single well-defined conformation in aqueous solution,[4] there is evidence that angiotensin II takes up a dominant conformation in nonpolar environments and within an antibody−hormone complex.[5−7] NMR studies of angiotensin II in water at 5 °C suggest a single, well-defined conformation for the hormone that shows similarities to the conformation of the peptide bound to a high-affinity antibody.[8]

The conformational and biological properties of many modified angiotensin II structures have been examined in attempts to define the dominant form of the hormone in solution and elucidate critical interactions in complexes of the hormone with receptors.[9−11] When the tendency of a small peptide to adopt a particular conformation is weak, a collection of interconverting conformations may be present in a sample. In these situations, the addition of fluoroalcohols to the solution may preferentially stabilize one or more of the conformations, especially if the peptide has an intrinsic tendency to take up a helical conformation.[12,13] The presence of trifluoroethanol (TFE) induces significant changes in CD spectra of angiotensin II and its analogues, suggesting the presence of such an effect.[14]

We have examined [val^5]angiotensin II (asp-arg-val-tyr-val-his-pro-phe), an analogue of angiotensin II in which valine at position 5 replaces the usual isoleucine residue. This analogue has been found to have the same biological and immunoreactive properties as endogenous angiotensin II.[15] Fermandjian et al. examined biological activity in a series of angiotensin II analogues and concluded that size and branching of the amino acid at position 5 are important in directing the Tyr4 and His6 residues in their interactions with cognate parts of the hormone's receptors.[16] Interactions of the peptide with TFE were explored as part of this study. There is evidence that fluorinated alcohols form relatively long-lived (∼1 ns) complexes with larger peptides;[17−20] we now report that TFE appears to form complexes with the smaller [val^5]angiotensin II.

Experimental Section

[val^5]angiotensin II was obtained in >95% purity as a white powder from Sigma-Aldrich and used as received. The material was hydrated and contained an unknown number of acetate counterions. For purposes of calculating concentration it was assumed that one water molecule and one acetate were present per mole of peptide. Trifluoroethanol-*1,1-d*$_2$ and undeuterated trifluoroethanol were obtained from Sigma-Aldrich and used without purification. Water used in sample preparation was glass-distilled deionized water.

CD Spectroscopy. CD measurements were performed with an AVIV-202 spectropolarimeter equipped with a Peltier temperature control system and a demountable quartz cell of 1 mm path length. Samples were measured at wavelengths between 190 and 250 nm with a 1 nm resolution and an integration time of 3 s. Spectra reported are an average of 16 scans and are baseline corrected. Sample temperatures were determined (±1 °C) with a calibrated thermocouple (Fisher Scientific). Samples were equilibrated at temperature for at least 30 min before a spectrum were recorded. The peptide concentration was determined by UV spectroscopy, using $\epsilon_{257.5} = 195$ cm^2 mmol^{-1}.[21]

NMR Spectroscopy. NMR spectra were collected at 500 MHz with a Varian INOVA instrument equipped with a Nalorac

* Address correspondence to this author. E-mail: gerig@nmr.ucsb.edu. Phone: 805-893-2113. Fax: 805-893-4120.

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H/F probe with a *z*-axis gradient coil. The signal from the deuterated methylene group of TFE-d_2 was used as a lock signal. Sample temperatures were determined by using a standard sample of methanol (Wilmad) and are believed to have been constant to better than ±0.1 °C during the course of an experiment and accurate to better than ±0.5 °C. The probe was de-tuned as necessary to avoid effects of radiation damping.

Samples for NMR experiments were ~5 mM in peptide. Apparent sample pH was determined by a Model IQ150 pH meter (IQ Instruments, San Diego, CA) equipped with a 4-mm o.d. stainless steel electrode. The reported pH was not corrected for the presence of fluoroalcohol or isotopic composition. Samples contained a trace of deuterated trimethylsilylpropionic acid (TSP, Stohler Isotope Chemicals) to provide a reference signal that was set to 0.0 ppm.

Vicinal (three bond) coupling constants ($^3J_{C\alpha HNH}$) to peptide hydrogens were estimated by deconvolution of the observed N−H line shapes, using the deconvolution feature of Vnmr software or the program PeakFit (SYSTAT, Richmond, CA).

Self-diffusion coefficients for sample components were determined by bipolar double stimulated echo pulsed field gradient experiments.[22] Samples were allowed to equilibrate in the probe at the regulated temperature at least 3 h before attempting diffusion measurements. Field gradient pulses were calibrated by using the diffusion coefficient of water in deuterium oxide.[23] Consideration of the reproducibility of the diffusion coefficients suggested that the values quoted below are reliable to at least ±15%. The conclusions reached in this paper are not dependent on high accuracy for the diffusion coefficients and it did not seem profitable to strive for greater accuracy.

Two-dimensional ^{1}H−^{1}H NOESY, TOCSY, and ROESY spectra were collected and analyzed as described previously.[19]

Solvent−peptide ^{1}H{^{19}F} intermolecular NOE data were obtained by using the pulse sequence described previously.[19] Peptide proton−solvent fluorine intermolecular cross-relaxation parameters (σ_{HF}) were estimated by the procedures previously described.[17,24,25] Signal intensities were determined for a range of mixing times ($50 < t_{mix} < 600$ ms) and the observed peak intensities fit to the empirical function $At_{mix} + Bt_{mix}^2$, with the coefficient A ($\equiv\sigma_{HF}$) being taken as the initial slope of the data. Corrections for incomplete inversion of the solvent fluorine resonance were applied. It was estimated that cross-relaxation parameters have accuracies of ±20−50%, depending on the S/N ratio of the signal under consideration.

Theoretical intermolecular cross-relaxation parameters (σ_{HF}) expected for a peptide proton immersed in a homogeneous mixture of trifluoroethanol and water were obtained by the methods described previously.[17,25] The radii of spheres representing TFE and TSP molecules were 2.46 and 3.62 Å, respectively, values obtained by the method described earlier.[19]

Dynamic viscosities of 40% TFE−water were estimated to be 3.68, 2.72, and 1.67 cP, respectively, at 5, 12, and 25 °C by interpolation of the kinematic viscosity data of Kaiser et al.[26] and use of the density data of Esteve et al.[27] The viscosities of a NMR sample may differ slightly from these values due to the presence of the peptide and TSP.

Molecular modeling and visualization were done with SYBYL (Tripos), PYmol (DeLano Scientific LLC), and MOLMOL.[28] The program DYANA was used to find conformations consistent with distance constraints from assigned ^{1}H{^{1}H} NOEs and observed $^3J_{C\alpha HNH}$ coupling constants.[29,30]

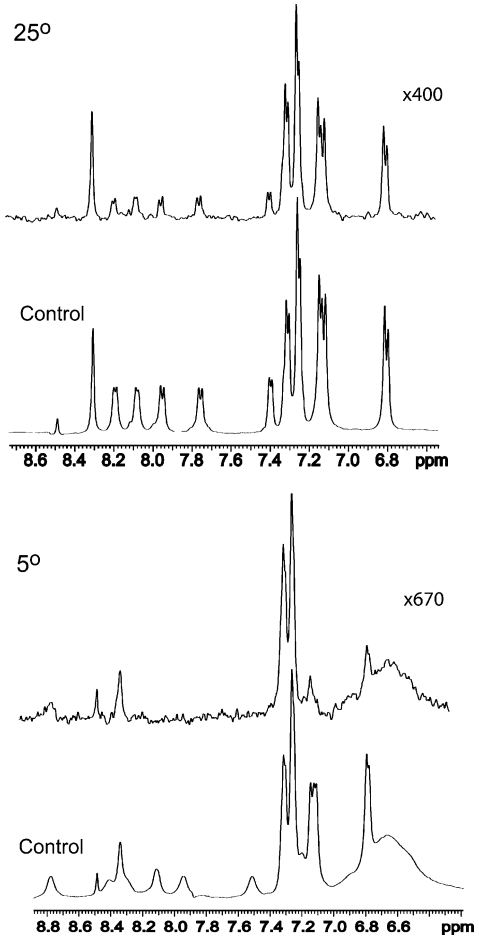

Figure 1. ^{1}H{^{19}F} intermolecular NOEs in the low field part of the spectrum of [val^5]angiotensin II at pH 5.6. The sample contained 40% trifluoroethanol-d_2, TSP as a reference signal (0.0 ppm), and an unknown amount of acetate as an impurity (1.99 ppm). The mixing time for the 25 °C NOE spectrum (top) was 600 ms while the mixing time for the 5 °C spectrum was 500 ms. The broad feature about 6.65 ppm in the 5 deg spectra arises from the alcohol proton of TFE, which is in slow to intermediate exchange at this temperature.

Results

Intermolecular Solvent−Peptide NOEs. Proton spectra of [val^5]angiotensin II were assigned by using TOCSY and NOESY data. The assignments made are listed in the Supporting Information. Overhauser effects on the proton signals of [val^5]-angiotensin II produced by inverting the magnetization of the fluorine spins of the TFE solvent molecules were determined at temperatures between 5 and 25 °C. Some representative results are shown in Figures 1 and 2. It is striking how many of the peptide signals exhibit near-zero solvent NOEs at 5 °C.

The initial rate of change of a ^{1}H{^{19}F} NOE with mixing time was used to estimate the cross-relaxation parameter σ_{HF} for interaction of solvent fluorines with a peptide proton. There were some overlaps in the proton spectrum of the peptide but values of σ_{HF} could be estimated for about 30 protons of the peptide. Values of σ_{HF} collected at 5 and 25 °C are given in the Supporting Information and are summarized graphically in Figures 3 and 4.

Structure of [val^5]angiotensin II. The dipole−dipole interactions that lead to the observed ^{1}H{^{19}F} intermolecular NOEs presumably are a result of collisions of solvent molecules with the solute peptide. All spins of the solute cannot be equivalently exposed to solvent molecules and the observed σ_{HF} presumably

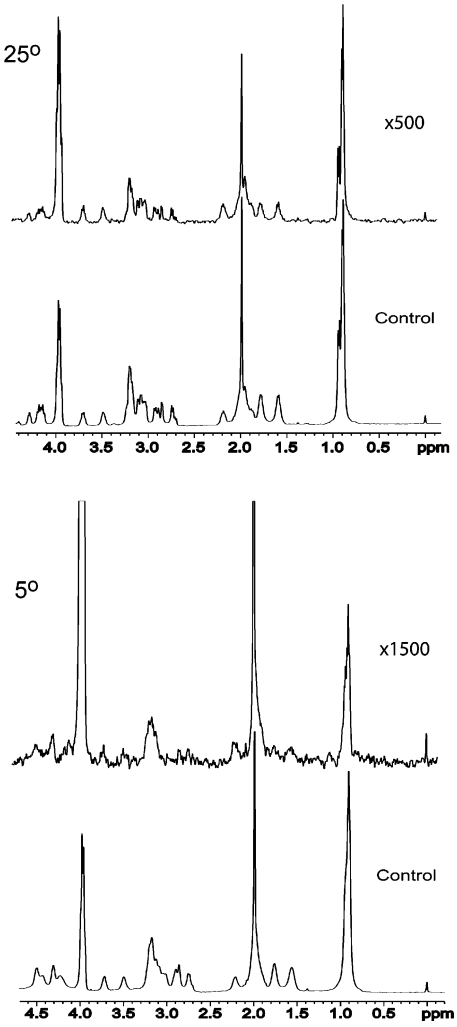

Figure 2. $^1H\{^{19}F\}$ intermolecular NOEs in the high field part of the spectrum of [val^5]angiotensin II in 40% TFE-d_2 at pH 5.6. The mixing time for the 25 °C NOE spectrum (top) was 600 ms while the mixing time for the 5 °C spectrum was 500 ms.

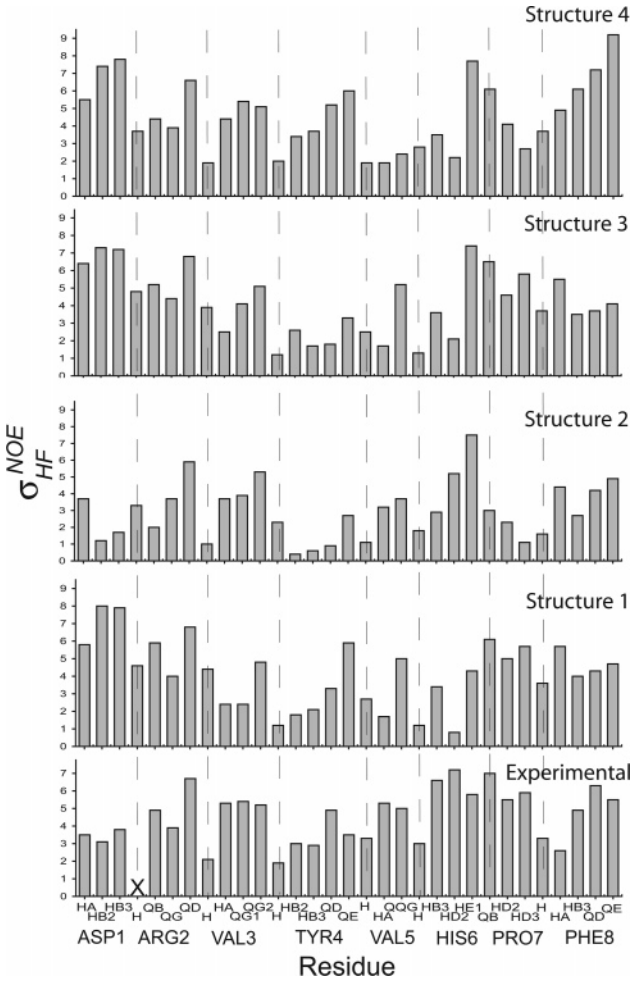

Figure 3. Cross-relaxation parameters (σ_{HF}) for interaction of fluorines with protons of [val^5]angiotensin II at 25 °C and pH 5.6. The sample contained 40% trifluoroethanol-d_2. The × indicates the Arg2 peptide proton. This signal is missing from the ^1H spectrum due to rapid exchange with the solvent at this temperature.

reflect in some manner the three-dimensional structure of the peptide. We, therefore, sought further information regarding this structure.

1. CD Spectroscopy. CD spectra of [val^5]angiotensin II in the presence and absence of TFE are shown in Figure 5. A number of analogues of angiotensin II at pH values above 4.5 exhibit negative ellipticities at 225 nm of about the same magnitude as was found for [val^5]angiotensin II in 40% TFE.[14] Such spectra have been interpreted as indicating the presence of β-structures for angiotensin II;[14,31] the extrema observed could further indicate the presence of β-turn conformations.[32,33]

2. *Coupling Constants*. Vicinal coupling constants ($^3J_{NH\alpha CH}$) were estimated at 5 and 25 °C. At the higher temperature, the NH proton signals were present as doublets and deconvolution of the observed line shapes provided firm values for the coupling constants. Signals for the peptide hydrogen of Arg2 were absent at 25 °C due to rapid exchange with water. At 5 °C only broad signals were observed for each NH proton and deconvolution gave less reliable values for $^3J_{NH\alpha CH}$. At 25 °C, $^3J_{NH\alpha CH}$ for Val3, Tyr4, Val5, His6, and Phe8 groups were 8.4, 7.9, 8.2, 7.3, and 7.0 Hz, respectively. At 5 °C, the coupling constant for Arg2 was approximately 6 Hz while the value for Val3 increased to 9.4 Hz, with the other coupling constants remaining essentially the same as was observed at the higher temperature. The magnitude of the coupling constants observed and their relative insensitivity to temperature are consistent with previous observations of angiotensin analogues.[10,34]

The coupling constant $^3J_{NH\alpha CH}$ presumably reflects the conformational angle ϕ. A fully extended β-structure for [val^5]-angiotensin II would be expected to exhibit vicinal coupling constants close to 9.7 Hz. The observed values of $^3J_{NH\alpha CH}$ thus indicate the presence of a conformation or conformations more folded than a fully extended β-structure.[35]

3. Diffusion. The translation diffusion constant of a solute is traditionally related to molecular dimensions by means of the Stokes−Einstein eq 1, where k_B is the Boltzman constant, T is the absolute temperature, η is the viscosity of the solvent, and r is the radius of a sphere representing the solute.[36] Equation 1 is most easily applied to estimating molecular sizes by comparing the diffusion of a species of interest to that of a reference material of known dimensions in the same solution.

$$D_{trans} = \frac{k_B T}{6\pi\eta r} \quad (1)$$

The experimental diffusion coefficients of [val^5]angiotensin II, acetate, and TSP in 40% TFE−water are given in Table 1.

The hydrodynamic radius of acetate ion in water is 2.26 Å.[37] Using acetate as a reference, it is estimated that the radius of [val^5]angiotensin II is 8 ± 1 Å. Essentially the same radius is

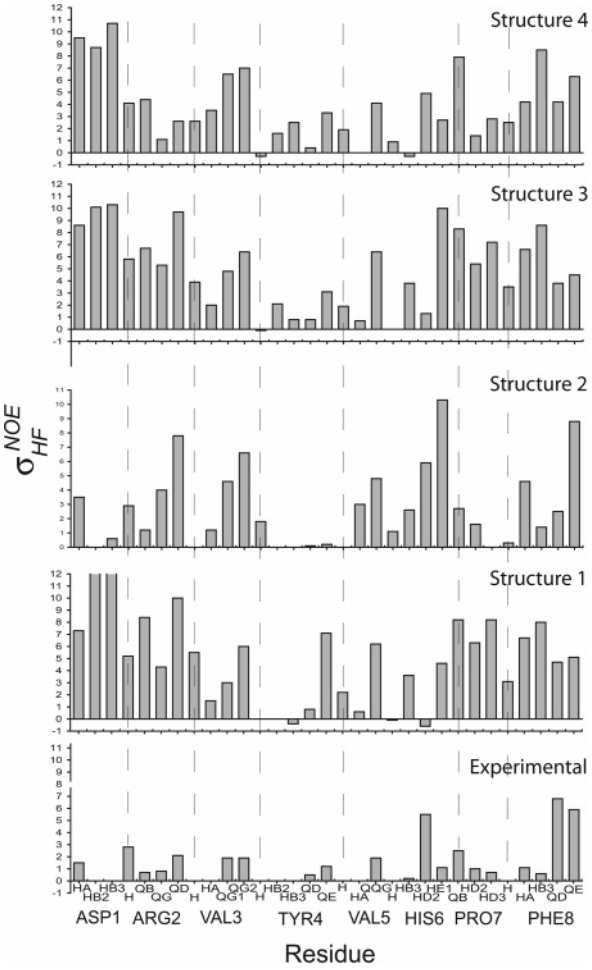

Figure 4. Cross-relaxation parameters (σ_{HF}) for interaction of fluorines with protons of [val^5]angiotensin II at 5 °C. The sample contained 40% trifluoroethanol-d_2.

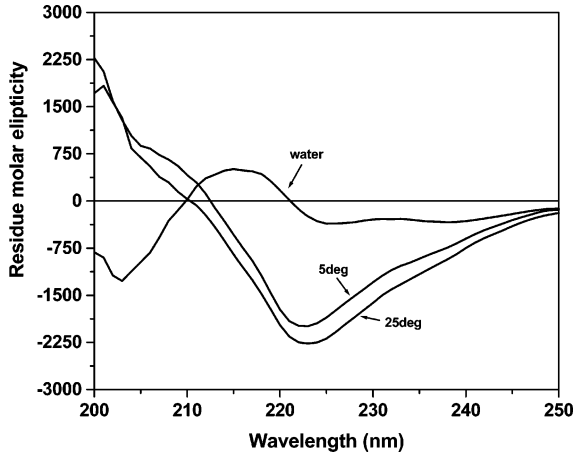

Figure 5. CD spectra of 0.4 mM [val^5]angiotensin II at pH 5.8 in water at 25 °C and in 40% trifluoroethanol at 5 and 25 °C.

obtained by using the TSP signal as a reference. This result is nearly the same as the molecular radius for angiotensin II derived from carbon-13 relaxation data.[38] As a fully extended β-structure of the peptide would be about 32 Å long, the conformation or conformations of [val^5]angiotensin II present in 40% TFE must be significantly folded.

4. Structure Determination from NMR-Derived Constraints. ^{1}H{^{1}H} NOESY data were collected for a sample of [val^5]-angiotensin in 40% TFE at 5, 12, and 25 °C to obtain distance constraints. Mixing times in these experiments were 100 ms or less in order to vitiate the effects of spin diffusion. NOEs observed in the spectra are described more fully in the Supporting Information. Fewer NOEs were observed at 12 and 25 °C than at 5 °C and our structural analysis focused on data obtained at the lowest temperature. Starting from approximately 300 assigned NOE cross-peaks observed at 5 °C, conformation-limiting constraints that included 16 intra-residue, 51 short-range (i, $i+1$) and 23 medium-range distances were obtained. Graphs indicating the distribution of these distance constraints are given in the Supporting Information. The upper distance limits, along with the observed $^3J_{NH\alpha CH}$ coupling constants and lower distance limits of 2.1 Å, were used as input to the program DYANA.[29] Approximately 1000 starting conformations were examined and ten structures for which the constraints were best satisfied were found. The backbone atoms of the ten structures had RMSD values of 1.97 ± 1.29 while the RMSDs for the heavy atoms of the structure were 3.37 ± 1.82, indicating that the structures obtained were not highly similar. These results are reminiscent of those reported for a similar NMR structural study of angiotensin II in 66% TFE.[7]

TABLE 1: Translational Diffusion Coefficients for [val^5]angiotensin II in 40% TFE-d_2 ($\times 10^6$ cm^2 s^{-1})

material	5 °C	12 °C	25 °C
[val^5]-angiotensin	0.518	0.904	1.32
trifluoroethanol-d_2	2.61	4.13	6.14
acetate	1.79	3.09	5.07
TSP	1.06	2.05	3.07

Since the molecular force field used in DYANA is intentionally crude, the "best" structures identified by DYANA were refined in the AMBER 4.1 force field[39] with the NMR distance constraints included. An implicit solvent with a dielectric constant of 63 was used and the force constant for the distance constraints was 30 kcal Å^2 mol^{-1}.[40] It was observed that starting structures characterized by the same DYANA target function value could converge under these conditions to dissimilar structures with dissimilar conformational energies.

Seven of the best ten DYANA structures had nearly the same target function value in the AMBER 4.1 force field and were similar to each other. The average of these structures, after being minimized in the AMBER force field, produced structure **1** shown below. The three remaining DYANA structures were similar and their average structure, after minimization, led to structure **2**. The DYANA structure with the best target function value was minimized to give structure **3**. All distance and coupling constant constraints were satisfied in all structures. However, structures **1** and **3** had all residues in most favored or "additionally allowed" regions of Ramachandran maps as judged by the program PROCHECK[41] and had similar conformational energies. Structure **2** had two residues in disallowed regions of Ramachandran maps and had a conformational energy higher than structures **1** or **3** by about 16 kcal/mol. It is seen from the representations in Figure 6 that structures **1**−**3** feature a somewhat curved backbone but differ in the orientations of the side chains.

A fourth structure of [val^5]angiotensin II was developed by starting with the lowest energy strcture of angiotensin II reported by Tzakos et al.,[8] changing residue 5 from isoleucine to valine, and minimizing the resulting structure in the AMBER 4.1 force field as described above. Stucture **4** had nearly the same conformational energy and estimated molecular radius as structure **1**. Structures **1** and **3** are similar to the structure of angiotensin II in water (as represented by structure **4**); the backbone RMSD when comparing structures **1**, **3**, and **4** was 2.0 Å.

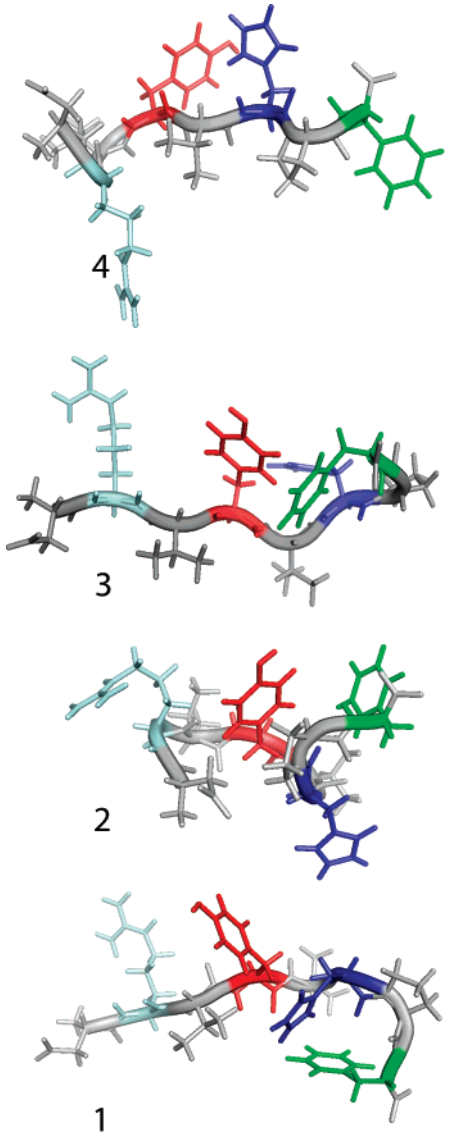

Figure 6. Structures of [val^5]angiotensin II at pH 5.6 in 40% TFE−water produced by consideration of intramolecular ^{1}H{^{1}H} NOEs observed at 5 °C and the $^3J_{NH\alpha CH}$ coupling constants. For clarity, the Arg2, Tyr4, His6, and Phe8 residues are rendered in cyan, red, blue, and green, respectively. Conformation angles for structures **1**−**4** are given in the Supporting Information.

Are any of these structures consistent with the observed ^{1}H-{^{19}F} intermolecular NOEs? We have described a numerical method that permits reasonably reliable estimates of solvent−solute cross-relaxation parameters.[25] This approach is based on a model in which all solvent−solute interactions are due to hard sphere collisions such that dipole−dipole interactions are modulated only by mutual diffusion. The method employs experimental translational diffusion coefficients, an estimate of the shape of the solute molecule based on its Connolly surface,[42] and the assumption that solvent molecules rotate rapidly enough that solvent spins can be regarded as being localized at the center of a representative sphere.[43] The procedure gives reasonable results: in the present work σ_{HF}^{NOE} for the TSP reference signal is predicted to within an average error of 30%, probably well within the experimental error of the determinations of σ_{HF}^{NOE} for this small signal.

Figure 3 compares values of σ_{HF}^{NOE} predicted for [val^5]-angiotensin conformations **1**−**4** at 25 °C. It is clear that neither the pattern nor the magnitude of experimental solvent cross-relaxation terms agree with the predicted values for any single structure. At 5 °C, the lack of agreement between observed and predicted σ_{HF}^{NOE} values is even more apparent (Figure 4). At the lower temperature, many ^{1}H{^{19}F} NOEs are near zero. Some σ_{HF}^{NOE} are predicted to be zero in a particular conformation but not all of them at the same time in a single structure.

It may be the case that the peptide exists as a mixture of equilibrating structures at these temperatures. Efforts to find linear combinations of the intermolecular NOEs of structures **1**−**4** were made but no combination of NOE spectra from these structures provided significantly better agreement with experiment than did the σ_{HF}^{NOE} values of a single structure.

We then sought to expand the "basis set" of conformations of [val^5]angiotensin II by a conformational search using the electrostatically driven Monte Carlo (EDMC) method with the ECEPP/3 force field.[44,45] Solvation effects were included by using the SRFOPT method.[46] The program ECEPPAK was used to explore the conformational space of the peptide at 278 K in the presence of NMR-derived medium- and long-range constraints. Starting with a random conformation of the peptide, a total of 320 energy-minimized conformations were generated, with 16 conformations finally accepted. By using RMSD agreement criteria, it was determined that these could be grouped into 3 families of conformations. Conformational energy minimization of these structures in the AMBER 4.1 force field led to structures that were no lower in energy than structure **1** and offered no apparent improvement in the ability to bring the predicted solvent−solute cross-relaxation terms for [val^5]-angiotensin II into better agreement with experimental observations.

It was concluded that the interactions of TFE molecules with the peptide giving rise to the observed NOEs cannot be described solely in terms of a model that involves diffusive, hard-sphere encounters of these species. Experimental and computational results in other systems have suggested that TFE preferentially accumulates near a peptide dissolved in a TFE−water mixture.[47] Since the contribution of a given TFE molecule to an observed σ_{HF}^{NOE} depends on the distance between the proton of interest and the TFE molecule, accumulation of TFE near the surface of a peptide would increase σ_{HF}^{NOE} relative to the value expected on the basis of collisional encounters in a medium in which the solvent components are homogeneously distributed. MD simulations of the [val^5]angiotensin II/40% TFE−water system indeed show the accumulation of TFE molecules near the peptide (Chatterjee, work in progrsss). Some of the σ_{HF}^{NOE} values that are larger than anticipated thus could be explained by this selective solvation model. However, σ_{HF}^{NOE} values appreciably *smaller* than those expected on the basis of the collisional encounter model, such as many of those found in this work at 5 °C, must be due to some other consideration.

Strongly negative peptide−fluoroalcohol intermolecular NOEs have been observed in several systems and have been explained in terms of the formation of long-lived complexes between the peptide and the fluoroalcohol.[17,19,20] If such complexes persist long enough, the dipolar interactions between peptide hydrogens and TFE fluorines become, in effect, intramolecular interactions, so that the motions modulating the spin dipole−spin dipole interactions are those associated with the rotational diffusion of the complex. Halle has shown that the contribution of such long-lived association of solute and solvent molecules leads to the expression for σ_{HF}^{NOE} given by eq 2[48]

$$\sigma_{HF\ complex}^{NOE} = \frac{1}{10}\gamma_H^2\gamma_F^2\hbar^2[-J'_2(\omega_H - \omega_F) + 6J'(\omega_H + \omega_F)] \quad (2)$$

with the spectral density function J'_2 defined as

$$J'_2(\omega) = \sum_{k=1}^{N} \frac{n_k}{r_k^6} \frac{\tau_{C,k}}{1 + \omega^2\tau_{C,k}^2} \quad (3)$$

The summation in eq 3 is taken over N classes of solvent molecules, each class containing n_k solvent spins at a distance r_k from the peptide proton of interest. Interactions are characterized by the correlation time $\tau_{C,k}$, defined by $1/\tau_{C,k} = 1/\tau_R + 1/\tau_{M,k}$, where τ_R is the rotational correlation time of the complex, taken here to be the same as the rotational correlation time of the peptide, and $\tau_{M,k}$ is the mean residence time of the spins of a class of solvent molecules in a complex.

By using a radius of 8.5 Å for [val^5]angiotensin II and the Stokes−Einstein equation, it is estimated that the rotational correlation time (τ_R) of the hormone is 1.4 and 0.6 ns respectively at 5 and 25 °C. The latter value is consistent with $\tau_R \approx 0.5$ ns at 32 °C estimated from carbon-13 relaxation times.[38] Assuming that $N = 1$ and $n_k = 3$ (corresponding to the three fluorines of a single TFE molecule), $r_k = 6$ Å, and these rotational correlation times lead to the calculated σ_{HF}^{NOE} complex values shown in Figure 7. The value of r_k used corresponds roughly to the distance from an aromatic hydrogen to the center of a TFE molecule interacting with the π-cloud of the aromatic ring or from a peptide hydrogen to the center of a TFE molecule hydrogen bonded to the peptide carbonyl group. Taking the calculated σ_{HF}^{NOE} values for structure **1** as a reference, it is seen that the experimental σ_{HF}^{NOE} values at 25 °C tend to be somewhat larger than expected (Figure 3) while at 5 °C experimental cross-relaxation terms are smaller than anticipated. Figure 7 shows that a mean residence time of ∼0.5 ns is consistent with these observations, with complex formation adding to the σ_{HF}^{NOE} due to hard-sphere interactions at 25 °C and subtracting from these values at 5 °C. Of course, the values of N and r_k chosen for the calculations are not unique and many combinations of these could produce the same result. The calculations behind Figure 7 merely show that a reasonable set of parameters for eq 3 can be consistent with our experimental results and support the conclusion that the formation of long-lived peptide−TFE complexes is an important aspect of the observed σ_{HF}^{NOE} values in the [val^5]angiotensin II−trifluoroethanol system.

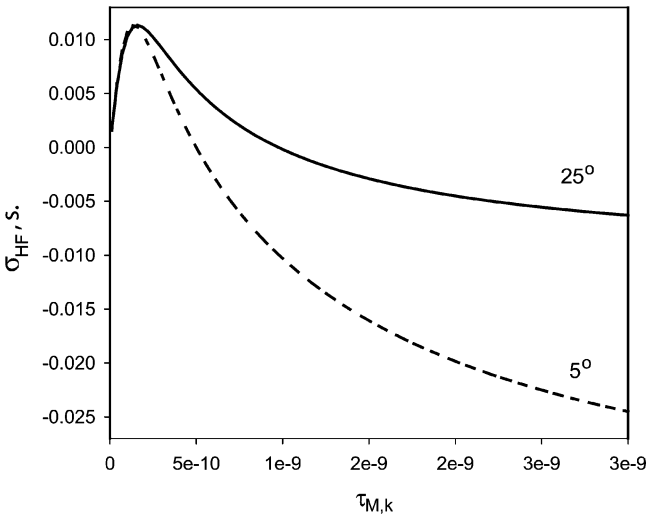

Figure 7. Calculated σ_{HF}^{NOE} for the "sticky collision" model described by eq 3. For the calculation, a single TFE molecule ($n_k = 3$) at a distance of 6 Å from the observed hydrogen was assumed. The curves differ primarily because the rotational correlation time (τ_R) is longer at 5 °C.

Discussion

Structural constraints available from NMR observations appear not to be consistent with a single unique conformation for [val^5]angiotensin II in 40% TFE−water. One could presumably fit coupling constant-dihedral angle and NOE-distance constraints to a family of conformations.[49] A procedure to select and evaluate the conformations used in such a calculation would be required. Typically, conformational energy defined by a molecular mechanics force field is used to score conformations, with those exhibiting low conformational energy forming the "basis set" for such a fitting exercise. Before such an approach could be productively applied to the system we examined, some considerations that would have to be resolved include determining how best to take into account the electrostatic interactions between the charged residues of the peptide and how to deal with the effects of a mixed TFE−water solvent on the conformational energies.[50] There have been attempts to deal with solvation of angiotensin II by water[51] in computations but for our system any approach to reckoning solvation energy would have to deal with the fact of a mixed organic−water solvent and the apparent tendency of TFE to aggregate nonhomogeneously around the peptide. Until reliable methods to rank energies of conformations (and more experimental structural constraints) become available, conclusions we have reached about structure(s) of the hormone must be regarded as provisional. It is likely, however, that a single conformation is not consistent with the available intermolecular NOE data.

An indication of the need for a more sophisticated treatment of electrostatic interactions and solvent effects on conformational energy is that, using our assumptions, conformations such as **2** were found to be appreciably higher in energy than conformation **1**, **3**, or **4** yet are more consistent with the molecular radius of [val^5]angiotensin II suggested by diffusion experiments. The agreement of σ_{HF}^{NOE} calculated from these structures with experimental observations was also somewhat better. However, such structures were more tightly folded than conformations like **1**, **3**, or **4**, and often were not fully consistent with Ramachandran maps or observed coupling constants.

It was our hope that determination of the cross-relaxation effects between solvent fluorines and protons of the peptide would lead to additional constraints on the structure of this peptide. This hope was predicated on the naive assumption that hydrogens on the surface of the peptide would be more exposed to interactions with bulk solvent while hydrogens on the interior would be protected from solvent interactions, leading to a reduction in dipolar interactions with solvent spins because of the distance dependence of these interactions. Indeed, the calculations of σ_{HF}^{NOE} shown in Figures 3 and 4 indicate that the contribution of diffusive hard sphere encounters to intermolecular $^1H\{^{19}F\}$ NOES between [val^5]angiotensin II and TFE are strongly dependent on three-dimensional structure of the peptide. We have now found evidence in several peptide−fluoroalcohol−water systems that the fluoroalcohol can form fluoroalcohol−peptide complexes within which proton−fluorine dipolar interactions can be significant contributors to observed σ_{HF}^{NOE} cross-relaxation parameters.[17−20] The magnitudes and signs of these contributions depend on structural details of the complex, which at present are unknown. Since an observed σ_{HF}^{NOE} may include these essentially intramolecular contributions

in addition to contributions from intermolecular dipolar interactions that are modulated by diffusion of solute and solvent in the bulk, it cannot be taken as an indication of solvent exposure.

The experimental σ_{HF}^{NOE} cross-relaxation data suggest that the charged side chains of [val^5]angiotensin II are sites of particularly strong interactions with TFE. At 5 °C, the σ_{HF}^{NOE} values of the side chains of Asp1, Arg2, Tyr4, His6, and Phe8 are significantly less than those expected for the low-energy peptide conformations (structures **1**, **3**, and **4**). Trifluoroethanol interactions at these side chains presumably take advantage of the polar and acidic properties of the fluoroalcohol. Hydrophobic interactions of the fluoroalcohol at Val3 and Val5 may account for the significantly reduced cross-relaxation terms for the side chains of these residues as well.

X-ray and light-scattering studies from several groups show that fluoroalcohol molecules cluster in water solution.[52−55] Aggregation of trifluoroethanol at 25 °C to give clusters with Stokes radii of approximately 6 Å takes place at a concentration of TFE similar to that used in the present work. Should such aggregation increase at lower temperatures it would, by withdrawing TFE molecules from interactions with dissolved peptide, lead to a reduction of TFE−peptide NOEs. Until studies of TFE aggregation under our sample conditions are available, we cannot rule out this possibility. However, light and small-angle X-ray scattering of aliphatic[56] and fluorinated aliphatic[52] alcohol−water mixtures show decreased scattering with decreasing temperature, presumably due to reduction in the number and size of alcohol−water aggregates present as the temperature is lowered.

Conclusions

Analysis of NOESY data indicates that the peptide hormone [val^5]angiotensin II in 40% TFE−water exists as a mixture of conformations. Intermolecular ^{1}H{^{19}F} NOE data were collected in the hope that that solvent−solute cross-relaxation effects so identified would provide additional constraints on the number and proportion of conformations present. Instead, evidence was developed that the fluoroalcohol molecules interact strongly enough with regions of the peptide that relatively long-lived (∼0.5 ns) complexes of the hormone and TFE are formed. The contributions of proton−fluorine interactions in these complexes to the observed cross-relaxation parameters cannot be estimated without detailed knowledge of the structures of the complexes. Thus, solvent−solute intermolecular NOEs have no value in defining peptide conformation. There is evidence that TFE interacts particularly strongly with the charged side chains of the peptide, although hydrophobic interactions with some side chains could also be present.

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Supporting Information Available: A listing of the proton chemical shifts for [val^5]angiotensin II at all temperatures examined and indications of the nature and distribution of the intramolecular proton−proton NOEs observed in the structural studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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