Threonine[6]-Bradykinin: Molecular Dynamics Simulations in a Biphasic Membrane Mimetic†

Maria Pellegrini[‡,§] and Dale F. Mierke*[,‡,||]

Gustaf H. Carlson School of Chemistry, Clark University, 950 Main Street, Worcester, Massachusetts 01610, Department of Organic Chemistry, University of Padova, Biopolymer Research Center, Via Marzolo 1, Padova, Italy I-35131, and Department of Pharmacology and Molecular Toxicology, University of Massachusetts, Medical Center, 55 Lake Avenue North, Worcester, Massachusetts 01655

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The natural peptide [Thr[6]]-bradykinin, Arg[1]-Pro[2]-Pro[3]-Gly[4]-Phe[5]-Thr[6]-Pro[7]-Phe[8]-Arg[9], has been conformationally examined by molecular dynamics simulations using a two-phase box consisting of H_2O and CCl_4 to mimic the micellar environment utilized in the ^{1}H-NMR studies. The different conformations generated from distance geometry calculations were refined with extensive molecular dynamics simulations. The resulting conformations provide additional structural insight into the differing biological activities of native bradykinin and [Thr[6]]-bradykinin, produced by the one conservative substitution Thr[6] for Ser[6]. In addition, the simulations give some indication of the interaction of the peptide with the biphasic, hydrophilic/hydrophobic environment of the micelle. Such information is vital given the accumulating data indicating that the peptide first interacts with the membrane before the membrane-bound receptor. The structures of membrane-bound [Thr[6]]-bradykinin developed here provide experimental support for the interaction of residues 7 and 8 with the core of the membrane-bound receptor and the N-terminus and C-terminal arginine interacting with the extracellular portion of the receptor.

Introduction

Linear oligopeptides, up to about 20 residues, do not generally form definite secondary structure elements in isotropic media, unless stabilized by specific sequences,[1,2] a high content of α,α-disubstituted amino acids,[3] or environmental effects of particular solvent systems.[4,5] Micellar systems have often been used in the NMR study of peptides[6−11] as a mimetic for the environment the peptide may experience when interacting with a membrane-embedded receptor.[12,13] A micelle presents a hydrophobic core, created by the long acyl chains, and a polar surface (negatively charged in the case of sodium dodecyl sulfate, SDS, micelles) that mimic the biphasic environment of a membrane. The relatively small size and fast reorientation of the micelle provide a medium favorable for NMR studies using standard high-resolution techniques.

In the preceding paper we describe the results of the NMR study and distance geometry (DG) calculations on the nonapeptide [Thr[6]]-bradykinin, [Thr[6]]-BK, in a solution of water and SDS micelles.[14] The determination of conformation and orientation of the peptide in the biphasic environment is of particular interest considering that the two known receptors of [Thr[6]]-BK belong to the family of G-protein-coupled receptors[15] that span the cellular membrane with seven α-helical segments. It has been proposed that the recognition of the peptide hormones by such membrane-bound receptors can be mediated by the statistically more favored preadsorption on the target cell membrane.[16,17] Besides reducing the dimensionality of the collision process for hormone receptor binding,[17−20] accumulation of the peptides on the lipid bilayer has been proposed to induce orientations and conformations which more properly meet the requirements of the receptor.[21−23]

Here we report the results from extensive molecular dynamics simulations of [Thr[6]]-BK in a biphasic H_2O/CCl_4 simulation cell.[24] The aim of these simulations is to further refine the conformation of [Thr[6]]-BK, as well as elucidate the orientation of the peptide at the interface boundary and the depth of intrusion into the bilayer. The initial DG calculations provided a fast and complete search of the conformational space accessible to the peptide and determination of all the structures compatible with constitutional and experimental restraints. The resulting structures are limited in that the DG algorithm is based solely on molecular connectivity (and experimental data) and ignores important energetic features (e.g., partial charges and Coulombic interactions, Lennard−Jones attractive forces). A refinement of the DG structures utilizing a full force field will allow for calculation of accurate energies (accurate with respect to the force field) and the possibility of a Boltzmann weighing of the different structures. Of course to obtain a meaningful result, the molecular dynamics calculations must be carried out with explicit solvent, the same solvent as utilized in the NMR study. Furthermore the simulation in the presence of the solvent allows for the examination of peptide/solvent interactions and, in the case of a biphasic systems, the orientation of the peptide at the interface and preference for the two phases.

Explicit membrane−water interfaces for MD simulations have been developed,[25−27] but they require an enormous amount of computer resources. The H_2O/CCl_4 two-phase box sacrifices the detailed description of the

† Abbreviations: DG, distance geometry; NMR, nuclear magnetic resonance; NOEs, nuclear Overhauser enhancements; MD, molecular dynamics; BK, bradykinin; SDS, sodium dodecyl sulfate.

* To whom correspondence should be addressed at Clark University. Tel.: (508) 793-7220. Fax: (508) 793-8861. E-mail: dmierke@clarku.edu.
‡ Clark University.
§ University of Padova.
|| University of Massachusetts.

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micelle−water interface; no charged or zwitterionic headgroups are simulated explicitly, which certainly play a significant role in the initial stages of the peptide interacting with the membrane. However, after the initial interaction, the overall biphasic, hydrophobic/hydrophilic character of the membrane is the most important aspect for the conformation and orientation of the peptide, and this macroscopic feature of a membrane is described by the mixture of the two immiscible solvents. Molecular dynamics simulations using H_2O/CCl_4, which can be run on standard workstations, are therefore especially suitable for the energetic refinement of peptide (and protein) structures utilizing NMR data collected in micellar milieu.

Experimental Procedures

MD simulations and interactive modeling were performed using *Discover* (Consistent Valence Force Field, CVFF91) and *Insight II* (Biosym Technologies Inc., San Diego) on SGI Indigo2 (R4400 150 MHz) and Indy (R4400 175 MHz) computers.

Simulations were carried out with different starting structures, containing either a βI-turn or βII-turn in the C-terminus, as observed from the DG calculations.[14] One structure from the ensemble calculations described in the previous article[14] was placed into a two-phase (H_2O/CCl_4) simulation cell ($x = 40$ Å, $y = z = 30$ Å, where y is the axis normal to the phase interface) using 3-dimensional periodic boundary conditions.

In addition, a third MD simulation beginning with the same starting conformation (containing a C-terminal βI-turn) but with a different orientation relative to the hydrophobic/hydrophilic interface was carried out. The long axis of the molecule was placed normal to the interface of the two phases to explore the reorientation of the peptide. The size of the two-phase box was increased to $x = y = z = 40$ Å for this simulation.

The first two simulations contained 560 H_2O and 103 CCl_4 molecules, while the third consisted of 981 and 199 molecules of H_2O and CCl_4, respectively. All ionizable groups were treated as charged species without inclusion of counterions. Except for CCl_4, treated as one atom, all atoms were treated explicitly. The Lennard−Jones parameters for H_2O and CCl_4 and the charges for H_2O were taken from Berendsen[28] and Rebertus.[29] The geometric mean was used for the parameters between unlike atoms. The details of the simulation cell are described in the article by Guba and Kessler.[24] For all the simulations a time step of 1 fs was employed. Neighbor lists for calculation of nonbonded interactions were updated every 10 steps within a radius of 14 Å. The actual calculation of nonbonded interactions was carried out up to a radius of 12 Å. No switching function was applied. The same distance restraints as in the DG calculations were applied with a maximum force of 10 kcal mol^{-1} $Å^{-1}$. Two initial cycles of energy minimization (1000 steps of steepest descent and 3000 steps of conjugate gradients, convergence criterion of 1 kcal/Å) were carried out with the solute fixed followed by 5 ps of conjugate gradients minimization with all the atoms free to move; the convergence criterion was 0.1 kcal/Å. The system was subsequently heated in cycles of 1 ps from 50 to 300 K, in steps of 50 K. After 4 ps of equilibration, structures were sampled every 500 fs for 200, 342, or 346 ps, for the three simulations, respectively. One iteration took approximately 1.2 s on a SGI Indy. The analyses of the structures for dihedral angles, angles, and distances distributions and rmsd values were performed using home-written Fortran programs.

Results and Discussion

Conformation and Orientation of [Thr^6]-BK in H_2O/CCl_4 (Starting from a structure with a βI-turn in 6−9). One of the structures obtained from the previous ensemble calculations and belonging to the family characterized by a β-turn of type I about Pro^7-Phe^8 was placed in the two-phase box.[14] The charged side chains of the arginine residues and the N-terminus were positioned in the aqueous solution and the aromatic rings of the phenylalanines anchored to the apolar CCl_4 phase. The starting orientation at the interface was chosen as a reasonable situation for the peptide, considering the favorable electrostatic interactions between solvent and solute.

Table 1. Backbone Dihedral Angles (Degrees), Calculated as the Average over the Last 200 and 342 ps of Dynamics, Respectively, for the Simulation of [Thr^6]-BK Starting from a βI- and a βII-Turn Structure

	βI		βII	
residue	ϕ	ψ	ϕ	ψ
Pro^2	−67.4	149.0	−65.2	153.6
Pro^3	−67.4	−61.8	−66.5	−61.3
Gly^4	98.9	74.0	118.5	70.1
Phe^5	−123.2	30.2	−144.2	66.5
Thr^6	−89.9	123.4	−117.6	141.4
Pro^7	−72.2	−32.8	−76.8	166.6
Phe^8	−64.7	23.6	68.7	−16.1

Table 1 summarizes the values for the backbone dihedral angle of [Thr^6]-BK, calculated as an average over the 400 structures sampled in the last 200 ps of dynamics. The structures do not present significant violations to distance restraints with the exception of the Pro$^7{}_{H\alpha}$−Phe$^8{}_{HN}$ NOE, which on average is in violation of 0.4 Å, the same violation previously observed in the DG structures.[14] The C-terminal βI-turn that characterized the starting structure is maintained throughout the simulation. The ϕ and ψ values for the relevant residues Pro^7 and Phe^8 exhibit small differences from the average values obtained from the DG calculations (Pro^7, $\phi = -69 \pm 9°$, $\psi = -46 \pm 7°$; Phe^8, $\phi = -58 \pm 33°$, $\psi = 3 \pm 24°$), with the MD values in closer agreement with the values for an ideal βI-turn. The average pairwise root mean square deviation (rmsd), using the heavy backbone atoms of all residues, between the MD structure with the lowest energy and the 94 DG structures is 2.7 Å; this is comparable with the rmsd observed for the ensemble of the DG structures (2.5 Å). Considering only the four residues of the βI-turn, the rmsd is only 0.79 Å (a pairwise rmsd of 0.76 Å is observed for the ensemble of DG structures).

A stable hydrogen bond between Thr$^6{}_{CO}$ and Arg$^9{}_{HN}$ is present during most of the MD simulation. The distance between the carbonyl oxygen of Thr^6 and the amide proton of Arg^9 during the simulation is plotted in Figure 1 together with the hydrogen bond angle O···H−N. It is significant that the HN of Arg^9 displays the lowest temperature coefficient in the peptide (−3.1 ppm/K); however, great care must be used in the interpretation of the amide temperature coefficients since they may be influenced by the micellar environment.

In Figure 2 a stereoview of the lowest energy structure shows the orientation of the molecule in the two-phase box. The residues in positions $i + 1$ and $i + 2$ of the βI-turn are deeply buried in the hydrophobic phase, whereas the amino acids i and $i + 3$ are anchored to the aqueous phase *via* their side chains. The side chain of Arg^9 has its positively charged end completely in the aqueous phase, while the polar hydroxyl of Thr^6 is near the interface.

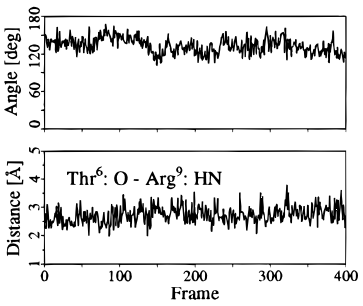

Figure 1. Time profile of an intramolecular hydrogen bond between Arg^9 and Thr^6: (top) the N−H···O angle between the amide nitrogen and proton of Arg^9 and the carbonyl oxygen of Thr^6 monitored over the 200 ps trajectory and (bottom) the distance between the amide hydrogen of Arg^9 and the carbonyl oxygen of Thr^6.

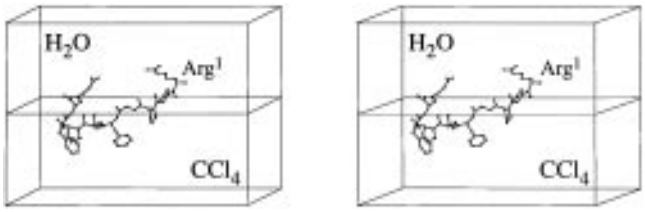

Figure 2. Stereoview of the lowest energy structure of [Thr^6]-BK in the biphasic H_2O/CCl_4 cell. Only the heavy atoms of the peptide are displayed for clarity. The phase interface is depicted.

The partitioning between the two phases is clearly illustrated by plots of the probability distribution function of selected atoms relative to the density profile. The atomic density profiles are defined as the average number of oxygen atoms (for H_2O) or carbon atoms (for CCl_4) in cross sections of thickness 0.5 Å in the direction parallel to the interface (the *y* axis), normalized to the atomic densities of liquid water and CCl_4, respectively. The probability distribution shows the average occupancy of cross sections of 0.1 Å in the *y* direction. As shown in Figure 3, the side chains of the two arginines are mainly exposed to the water phase, and the aromatic rings of Phe^5 and Phe^8 show a clear preference for the CCl_4 phase, while the hydroxyl group of Thr^6 populates the interfacial region. The Cγ of Pro^7 (residue $i + 1$ in the β-turn) is completely embedded in the apolar phase. The positioning of the β-turn about residues 7 and 8 places the atoms involved in the Thr^6_{CO}-Arg^9_{HN} hydrogen bond very near to the interface, slightly shifted toward the CCl_4 phase (Figure 4).

The formation of solvation shells can be characterized by radial distribution functions (rdfs), $g_{xy}(r)$, which give the probability of finding an atom of type *y* at a distance *r* from the atom of type *x*. The rdfs of the amide proton of Arg^9 and of the carbonyl oxygen of Thr^6 and Phe^8 relative to water oxygen were calculated (Figure 4). The rdfs were normalized by dividing the number of water oxygens by the bulk number densities. The water-exposed protons show a typical maximum in the distribution at the distance for a hydrogen bond with water, and the integrated area of the peak gives the number of water molecules in the first solvation cell. The three atoms analyzed occupy on average a similar position relative to the interface H_2O/CCl_4. It is evident that the atoms involved in the intramolecular H-bond, Thr^6_{CO} and Arg^9_{HN}, are shielded from the solvent, while the rdf of Phe^8_{CO} is consistent with the projection of the carbonyl into the solvent and formation of an intermolecular hydrogen bond with H_2O. Integration of the peak indicates 50% occupancy of a H-bond with the solvent, in agreement with the interfacial location of the atom examined.

Reorientation of [Thr^6]-BK in H_2O/CCl_4 (Starting from a structure with a βI-turn in 6−9). The same starting structure utilized in the preceding simulation was placed in a two-phase box with an orientation that is intuitively 180° "out of phase"; the N-terminus and the charged side chain of Arg^1 are in the CCl_4 phase, and the hydrophobic side chains of the phenylalanines are soaked in water. The aim of this second simulation was to explore different orientations of the peptide relative to the interface and the effect of the simulation conditions on the distribution of the solute between the two phases.

The reorientation process is clearly illustrated by the time course of the *y* coordinate of selected atoms compared with the density profile of the two solvents H_2O/CCl_4 (Figure 5). One of the protons at the amino terminus, which was initially deeply embedded in the CCl_4 phase and belongs to a charged group, travels a great distance in the process of reorientation; an additional example of the reorientation, but in the opposite direction (i.e., H_2O to CCl_4), is the time profile of $Phe^8_{C\gamma}$. The $Phe^5_{C\gamma}$ atom covers a much smaller distance. This is expected since Phe^5, in the middle of the peptide sequence, began closer to the H_2O/CCl_4 interface. The reorientation process is surprisingly rapid, requiring only 100 ps of dynamics. At frame number 200, corresponding to 100 ps of dynamics, the charged groups have already entered the water phase and the phenylalanine side chains are within the hydrophobic CCl_4 medium.

The structures obtained during the last 200 ps of the simulation (i.e., after the reorientation and the stabilization at this new position) are characterized by the same C-terminal folding of the structures obtained from the previous simulation; the dihedral angles for the residues in positions $i+1$ and $i+2$ of the turn are: Pro^7, $\phi = -69 \pm 9°$, $\psi = -35 \pm 9°$; Phe^8, $\phi = -65 \pm 9°$, $\psi = 27 \pm 22°$. Furthermore the hydrogen bond characterizing the β-turn is maintained during the reorientation process with only small fluctuations, as indicated by the plot of the distance Thr^6_{CO} −Arg^9_{HN} and of the H-bond angle O···H−N during the 346 ps of the simulation (Figure 6).

The final orientation of the molecule relative to the interface obtained from the two simulations is almost identical. The time scale of the MD simulations is definitely too small to calculate the partition coefficient of the solute between the two phases. No experimental restraints, or additional terms in the force field, are applied to define the orientation or the depth of intrusion of the molecule; therefore, the minimum to which both simulations converge can be a local minimum that can not be overcome during the simulation period. However, it is important to note that the same final distribution is obtained after reorientation, starting from significantly different initial conditions.

Conformation and Orientation of [Thr^6]-BK in H_2O/CCl_4 (Starting from a structure with a βII-turn in 6−9). The starting structure for the simulation was chosen from the family of structures with a β-turn of type II about Pro^7-Phe^8, obtained from the ensemble calculations.[14] The molecule was placed in the two-

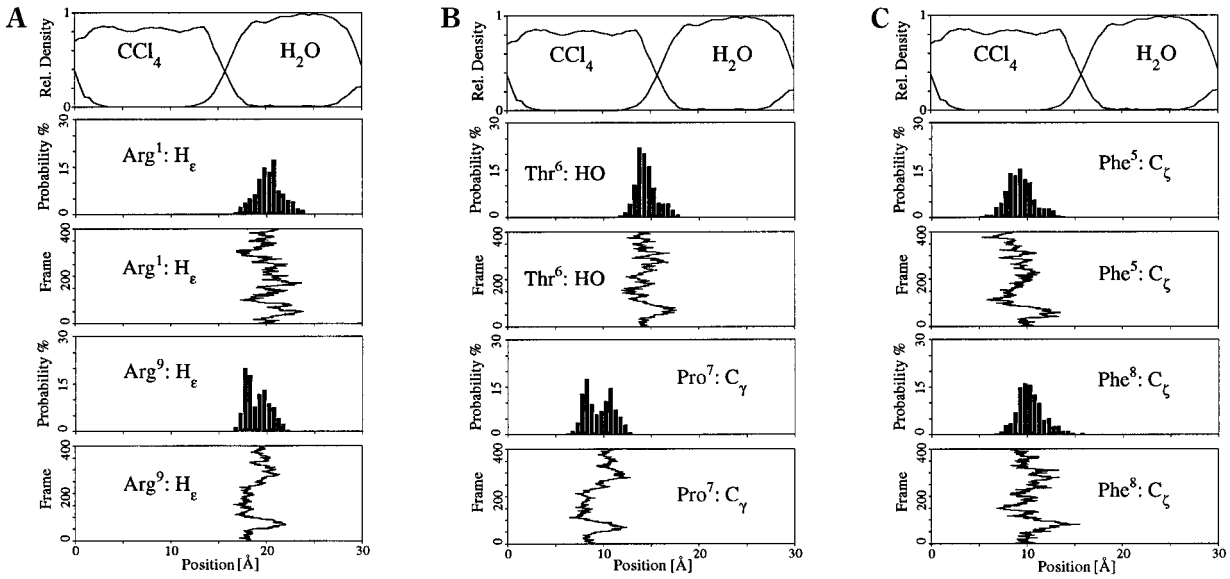

Figure 3. Probability distribution and time course of the *y* coordinate of selected atoms of [Thr^6]-BK referred to the density profile of H_2O/CCl_4. The data are relative to the simulation of a structure containing a βI-turn, starting from a favorable orientation (see text for details).

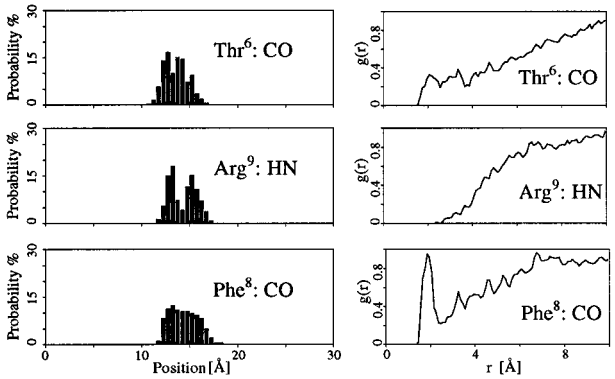

Figure 4. Probability distribution of the *y* coordinate in reference to the density profile of H_2O/CCl_4 (left) and radial distribution functions with water oxygens of selected atoms (right). The data are from the simulation of a βI-turn-containing structure, starting from a favorable orientation (see text for details).

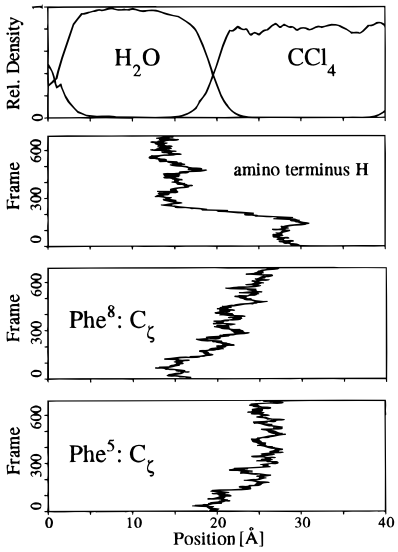

Figure 5. Time course of the *y* coordinate of exemplary atoms of [Thr^6]-BK in reference to the density profile of H_2O/CCl_4. The data illustrate the rather quick reorientation of the molecule during the first portion of the 346 ps simulation.

phase box in an intuitively favorable orientation (i.e., hydrophilic residues in H_2O and hydrophobic residues in the CCl_4 phase), as with the βI-turn structure described above.

The backbone dihedral angle values averaged over the final 342 ps of dynamics are reported in Table 1. The distorted βII-turn in the C-terminus of the molecule is maintained during the simulation. The analysis of the NOE restraint violations reveals that the largest deviations (>0.4 Å) involve distances between $Phe^8{}_{HN}$ and protons in the preceding residues ($Pro^7{}_{H\gamma}$ and $Thr^6{}_{CH_3}$). These violations can be explained by considering the "flip" in the orientation of the amide bond between residues 7 and 8 upon going from a βI- to a βII-turn: The new orientation of $Phe^8{}_{HN}$ fulfills the $Pro^7{}_{H\alpha}$–$Phe^8{}_{HN}$ NOE but at the expense of violating the NOEs between $Phe^8{}_{HN}$ and Pro^7/Thr^6. Nevertheless in the time scale of the simulation carried out, with the requirement that the structure fulfills *all* the NOEs simultaneously (in contrast to the ensemble calculations described in the preceding article), the βII-turn structure is stable and no unfolding or conversion to a βI-turn structure is observed. The hydrogen bond found in the βI-turn structure is not well defined in this case. The average distance between $Thr^6{}_{CO}$ and $Arg^9{}_{HN}$ is 3.2 Å (±0.4), with an angle O···H−N of 146° (±11). The orientation of the molecule within the two-phase box is similar to the one observed from the other two simulations, and it is illustrated in Figure 7 using the lowest energy structure.

Conclusions

Exemplary structures obtained from the study of [Thr^6]-BK in H_2O/SDS by NMR distance geometry and ensemble calculations were refined by molecular dynamics calculations in a mimetic of the micellar environment. Both structures, characterized respectively by a C-terminal β-turn of types I and II, revealed to be stable in the biphasic environment used in the simulation. The NOE violations present in the two cases were tolerated and did not induce unfolding or transitions to different secondary structures. This supports the hy-

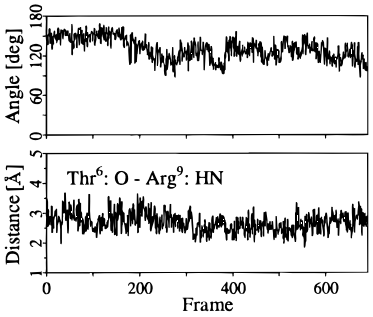

Figure 6. Time profile of an intramolecular hydrogen bond between Arg^9 and Thr^6 from the trajectory beginning with the structure βI-turn in an unfavorable orientation (see text for details): (top) the N−H···O angle between the amide nitrogen and proton of Arg^9 and the carbonyl oxygen of Thr^6 monitored over the 342 ps trajectory and (bottom) the distance between the amide hydrogen of Arg^9 and the carbonyl oxygen of Thr^6.

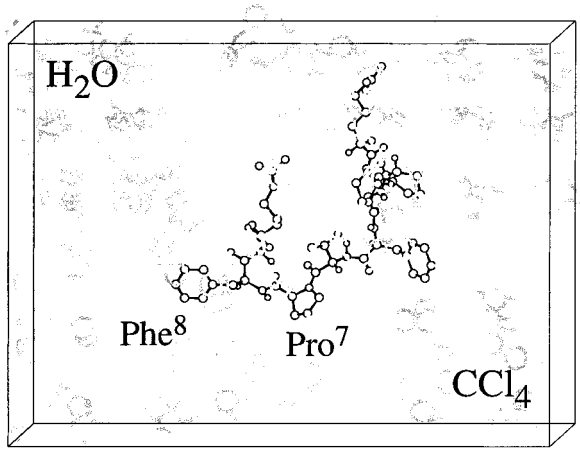

Figure 7. Orientation of [Thr^6]-BK in the two-phase box, as resulting from a 342 ps simulation of a structure containing a βII-turn in Pro^7-Phe^8. The peptide is represented using only heavy atoms, the H_2O molecules with all atoms, and the CCl_4 molecules as one atom.

pothesis of the existence in solution of the two families of structures as favored conformations. The existence of the NOE violations confirms that one set of structures alone can not account for the experimental data; only by averaging over both families of structures can the NMR observables be reproduced.

The simulation in the H_2O/CCl_4 box established the orientation of the molecule at the interface and the depth of intrusion in the hydrophobic phase. As expected the βI- and βII-turn structures assume a very similar arrangement, with the charged terminal amino acids anchored to the aqueous phase and the aromatic ring of Phe^5 and the two central residues of the turn embedded in the CCl_4. The description of a correct phase preference was further confirmed by the rapid reorientation of the molecule when placed in the two-phase box in a different (unfavorable) starting position, with a decided convergence to the same orientation of the other two rationally biased simulations. Even in the approximation of the micelle with a simple two-phase box (no explicit simulation of the charged headgroups at the interface), the structure-enhancing effect of the SDS micelles on the linear nonapeptide can be understood considering that the β-turn structure allows for the charged residues to interact with the polar solvent while the hydrophobic Phe^5 and the central residues of the turn are embedded in the apolar environment of the micelle core.

The conformation and orientation of [Thr^6]-BK within the two-phase environment are consistent with the previously proposed important role of residues 7 and 8 in biological activity and with the potential of the chirality of residue 7 to control agonist and antagonist responses. The two residues in the β-turn appear to be oriented toward the membrane-embedded receptor, as proposed in the model of BK bound to the rat B2 receptor of Kyle,[30] and can therefore be responsible of specific interactions. In addition, the side chain of Phe^5 is described in the model as extending into hydrophobic clefts between transmembrane helices, in agreement with the orientation determined by the MD simulations. Finally, the C-terminal β-turn allows the arginine residues to be oriented toward the polar water phase: The N-terminal arginine and the free N-terminal amino group are essential for high-affinity binding and are thought to protrude into the extracellular phase and interact with negatively charged aspartic acid residues in the third extracellular loop of the receptor.[30]

The MD simulation in the presence of a mimetic of the micellar environment therefore provides an accurate description of the molecule in the biphasic system but requires a preliminary extensive search of the conformational space, with distance geometry and ensemble calculations, to assure adequate sampling and provide reasonable starting structures.

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