

Molecular dynamics simulations of peptides from BPTI: A closer look at amide–aromatic interactions

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Summary

Molecular dynamics (MD) simulations of short peptides in water were performed to establish whether it is possible to reproduce experimental data from chemical shift measurements by nuclear magnetic resonance spectroscopy. Three different tetrapeptides were studied. The first, YTGP (Tyr-Thr-Gly-Pro), shows an electrostatic interaction between the aromatic ring of Tyr and the backbone amide hydrogen atom of Gly. The second, YTAP (Tyr-Thr-Ala-Pro), cannot make such an interaction because of steric hindrance of the Ala side chain and hence does not show a well-defined conformation. The third, FTGP (Phe-Thr-Gly-Pro), is shown to alternate between two different conformations. It is demonstrated that small differences in chemical shift, corresponding to these slightly different conformations, can be quantitatively modeled in MD simulations when using the proper force-field parameters and water model. Explicit inclusion of hydrogen atoms on the aromatic rings is essential for a proper description of electrostatic interactions, but the choice of the water model is equally important. We found that a combination of the SPC/E water model and a revised GROMOS87 force field gives close agreement with experiment, while the same and other force fields in combination with SPC or TIP3P water did not reproduce the NMR data at all. Simulations of a longer peptide from bovine pancreatic trypsin inhibitor, containing the YTGP sequence, did show the interaction between the aromatic ring and the amide hydrogen, but not as pronounced as the simulations of shorter peptides.

Introduction

Nuclear magnetic resonance studies have shown that in a four-residue peptide from bovine pancreatic trypsin inhibitor (BPTI) in aqueous solution a non-random conformation can be found (Kemink et al., 1993). This peptide, with sequence Tyr-Thr-Gly-Pro (YTGP, residues 10–13), shows an upfield shift of 1.5 ppm for the Gly amide proton, which can only be explained by the ring current of the Tyr side chain. Similar synthetic peptides display a significantly different chemical shift: Phe-Thr-Gly-Pro (FTGP) shows an upfield shift of 0.7 ppm for the Gly amide proton, while Tyr-Thr-Ala-Pro (YTAP) does not show any shift change in the Ala amide proton as compared to random coil values (Bundi and Wüthrich,

1979; Merutka et al., 1995). NMR data are also available on the peptide comprising the first 15 residues of BPTI (P₁₋₁₅), which contains the YTGP peptide (Kemink et al., 1993; Kemink and Creighton, 1993). In this case the upfield shift of the Gly¹² amide proton is 1.8 ppm, slightly more than in the YTGP peptide and considerably more than in the intact protein, where the upfield shift of the Gly¹² amide proton is 1.2 ppm (Van Mierlo et al., 1991).

These NMR results have led to more investigations on the importance of aromatic groups for the stability of BPTI (Kemink and Creighton, 1995; Worth and Wade, 1995). Worth and Wade (1995) found, using molecular mechanics (MM) calculations, that the amide–aromatic interaction has two pronounced energy minima. The lowest energy in vacuo corresponds to an interaction

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Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; P₁₋₁₅, peptide representing the first 15 residues of BPTI; MD, molecular dynamics; MM, molecular mechanics; RDF, radial distribution function.

where the amide group is perpendicular to the ring, in agreement with results from *ab initio* calculations (Cheney et al., 1988; Mavri et al., 1993). Another minimum in vacuo is found for the interaction where the amide group is parallel to the ring. In solution or in a protein environment, the second minimum is energetically more favorable, because it allows the amide groups to interact with solvent or with other groups simultaneously. Crystallographic database studies confirm that the parallel orientation is more abundant than the perpendicular one in proteins (Mitchell et al., 1994).

Hitherto, the dynamic properties of these small peptides have not been studied experimentally. Nevertheless, on the basis of the chemical-shift difference between Gly³⁷ in BPTI, which has a strong interaction with Tyr³⁵ resulting in a chemical shift of 4.3 ppm for the proton (Tüchsen and Woodward, 1987; Van Mierlo et al., 1991), and Gly in the YTGP peptide, a two-state model was proposed that assumes the existence of an 'open' and a 'closed' form of the YTGP peptide. Kemmink et al. (1993) propose that the number of closed YTGP peptides is 44% in equilibrium. Since there is no direct experimental evidence to support this, and to be able to understand the different chemical shifts for YTGP, FTGP and YTAP in more detail, we performed molecular dynamics (MD) simulations of these peptides and of the longer P₁₋₁₅.

It is not at all evident that force-field-based methods are sufficiently accurate to reproduce the subtle interactions between aromatic rings and amide groups. Therefore, we have to establish whether it is possible to do this before we can interpret the MD trajectories. We used three force fields and three different water models, but we want to stress that it is not our intention to fit force-field parameters to the NMR data. The intention of this paper is twofold, i.e., first we want to establish whether it is possible to reproduce chemical shift data from NMR experiments by MD simulations, and second, if we can reproduce these data, we will try to explain the different behavior of the tetrapeptides in structural and dynamic terms. By means of simulations of the longer P₁₋₁₅ peptide we can assess the implications of our results for protein simulations.

Materials and Methods

For the three peptides YTGP, YTAP and FTGP, starting conformations were generated using Quanta 3.3 (MSI, 1994). These starting configurations were linear, corresponding to an all-trans backbone configuration, and each had N-terminal acetyl and C-terminal amide groups. For the starting structure of P₁₋₁₅ (sequence RPD-FSLEPPYTGPSK), the N-terminal part of BPTI, we used the first 15 residues from the crystal structure (Wlodawer et al., 1987; pdb entry 6PTI) with a free amino terminus and an amide group at the carboxyl terminus. In each case the structures were solvated in SPC water, using a cubic box containing 216 equilibrated SPC water molecules as a building block. The peptide volume was cut out of the water configuration by removing all water molecules within 0.23 nm of any peptide atom. This resulted in around 820 water molecules for the tetrapeptides and 2061 for P₁₋₁₅.

We used three different force fields for our simulations:

(i) G-93: the GROMOS87 force field with modified carbon-OW interaction parameters (Van Buuren et al., 1993; Mark et al., 1994; Daura et al., 1996).

(ii) G-94: the same as [G-93], plus explicit hydrogens on the C atoms of the aromatic rings (King, P.M., Mark, A.E. and Van Gunsteren, W.F., private communication, 1993).

(iii) OPLS: basically the one described by Jorgensen and Tirado-Rives (1988) plus the extension for hydrogens on aromatic rings as described by Jorgensen and Severance (1990), but we made use of the bonded parameters of GROMOS87 instead of AMBER (Weiner et al., 1986).

All three force fields were used with the SPC (Berendsen et al., 1981), SPC/E (Berendsen et al., 1987) and TIP3P (Jorgensen et al., 1983) water models. The charges and Lennard-Jones parameters for water models as well as aromatic rings are listed in Table 1. The modified carbon-OW parameters can be deduced from Table 1 using geometric mixing rules. For the simulations with SPC/E water and TIP3P water the same starting conformations were used as for the simulations with SPC. Although an equilibrated box of SPC water probably does not represent an equilibrated box of SPC/E or TIP3P

TABLE 1
FORCE-FIELD PARAMETERS FOR RING CARBON AND HYDROGEN ATOMS AND WATER MODELS

Force field	q (e)		σ (nm)		ε (kJ mol ⁻¹)		Water model	q (e)		σ (nm)		ε (kJ mol ⁻¹)	
	C	Hc	C	Hc	C	Hc		Ow	Hw	Ow	Hw	Ow	Hw
G-93	0.0	-	0.3741	-	0.5026	-	SPC	-0.82	0.41	0.316557	0.0	0.65019	0.0
G-94	-0.14	0.14	0.3741	0.237	0.5026	0.1187	SPC/E	-0.8476	0.4238	0.316557	0.0	0.65019	0.0
OPLS	-0.115	0.115	0.355	0.242	0.1256	0.02931	TIP3P	-0.834	0.417	0.315061	0.0	0.63639	0.0

The table lists charges and Lennard-Jones σ and ε values. TIP3P water has the experimental geometry (Ow-Hw=0.09572 nm, angle Hw-Ow-Hw=104.52°); SPC and SPC/E have a tetrahedral geometry (Ow-Hw=0.1 nm, angle Hw-Ow-Hw=109.47°). In the OPLS simulations, third neighbor interactions were scaled by 0.5. A cutoff of 1.0 nm was used in all cases, based on geometric centers of charge groups (groups of atoms with neutral charge). In the OPLS simulations we used the bonded parameters of GROMOS87 rather than AMBER (Weiner et al., 1986).

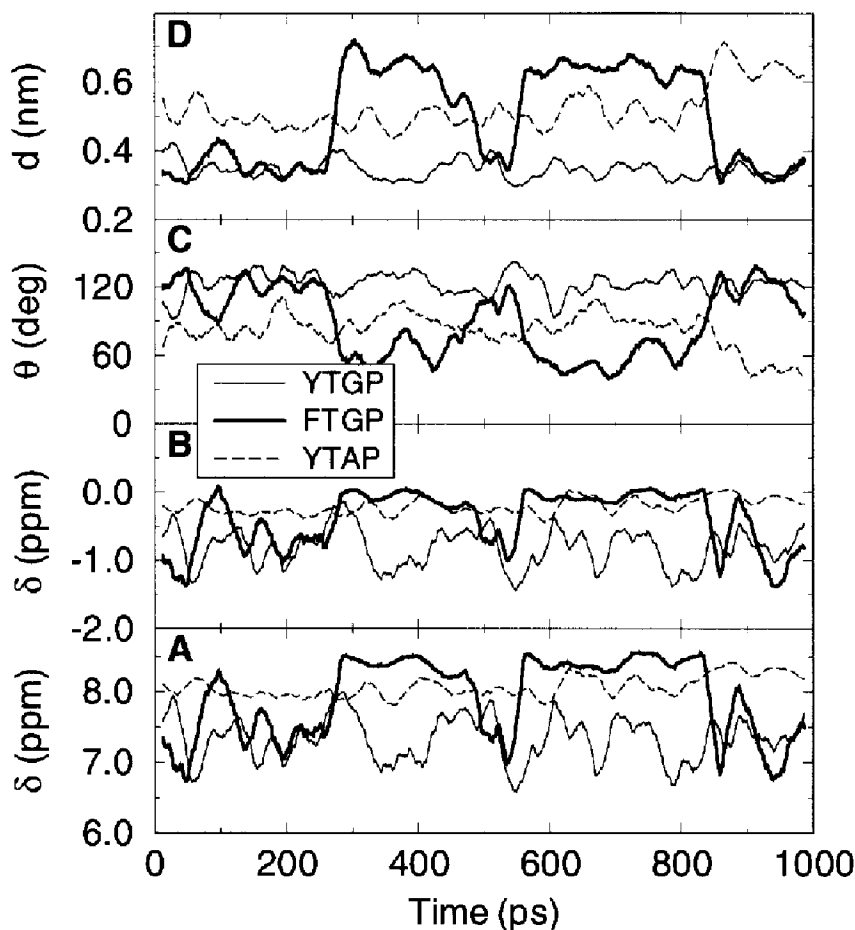


Fig. 1. (A) Chemical shifts of Gly-NH in YTGP and FTGP and of Ala-NH in YTAP, all in the G-94 force field with SPC/E water. (B) Contribution of ring current to the chemical shift of Gly-NH in YTGP and FTGP and of Ala-NH in YTAP, all in the G-94 force field with SPC/E water. (C) Angle between Gly/Ala-NH and the aromatic ring of Tyr/Phe. The angle θ is defined such that $\theta = 180^\circ$ corresponds to the NH vector pointing towards the ring. The ring is defined as the plane spanned by the CD1, CD2 and CZ atoms. (D) Distance between Gly/Ala-NH and the center of the aromatic ring. All data are shown as a running average over 25 ps.

water, the difference between the water models is small and equilibration will probably not take more than a few picoseconds.

The starting structures were energy minimized (steepest descents) for 100 steps. Then, initial velocities were taken from a Maxwellian distribution at 271 K, which is the temperature used for the NMR experiments. Although this temperature is somewhat lower than that used for deriving the force-field parameters, recent simulation work has shown that e.g. the self-diffusion constant of SPC/E water for temperatures above 250 K is in good agreement with experimental data (Báez and Clancy, 1994). The MD simulations were performed using temperature and pressure coupling to reference baths of 271 K and 1 bar, respectively, with coupling time constants $\tau_T = 0.1$ ps and $\tau_p = 0.5$ ps (Berendsen et al., 1984). Peptide and solvent were independently coupled to the heat bath. All covalent bond lengths as well as the water angle were constrained using SHAKE with a relative tolerance of 10^{-4} (Ryckaert et al., 1977), allowing a time step of 2 fs. Neighbor lists were updated every 20 fs. The Lennard-

Jones and Coulomb interactions were truncated using a single cutoff of 1.0 nm. The truncation criterion was based on charge groups, i.e., groups of atoms with integral charge. Interactions were calculated whenever the distance between the centers of geometry of the charge groups was less than the cutoff distance. It is necessary to state here that this criterion is slightly different from the one used in OPLS (Jorgensen and Tirado-Rives, 1988). However, we think that this method gives rise to a more spherical cutoff than the original OPLS method and is therefore preferable. In the GROMOS simulations, the van der Waals repulsion was reduced for 1-4 interactions, whereas the dispersion and Coulomb terms used the full interaction. In the OPLS simulations, all 1-4 interactions were scaled by a factor of 0.5 for repulsion, dispersion and Coulomb terms. Periodic boundary conditions were used in all three spatial dimensions. All MD runs were performed for 1.0 ns.

We used the GROMACS package (Van der Spoel et al., 1996), a parallel MD implementation that employs the same potential function as GROMOS87 and OPLS.

This software runs on multi-processor parallel computers, which were designed in our laboratory (Bekker et al., 1993; Berendsen et al., 1995).

Calculation of chemical shifts

To be able to compare our simulation data to experimental data, we need a method to compute chemical shifts from a structure. For this purpose we have used the empirical method of Williamson and Asakura (1993). Recently there has been considerable progress in the computation of chemical shifts from structural data (Ösaspay and Case, 1991,1994; Spera and Bax, 1991; Williamson and Asakura, 1993; Case, 1995). Although there is impressive progress in the ab-initio-based methods (de Dios et al., 1995; Pearson et al., 1995), they are not practical for our purpose; we want to average the chemical shift over our trajectory of 2000 frames, therefore the computation time must be short.

Results

Analysis of peptide conformations

Chemical shifts were computed using the 'total' program of Williamson and Asakura (1993). In Fig. 1A the chemical shifts (in ppm) of the Gly³-H protons in YTGP and FTGP and of the Ala³-H proton in YTAP from the simulations with the G-94 force field and SPC/E water are plotted as a function of time. The average chemical

shift for Gly³-H in YTGP is 7.3 (exp. 7.0), in FTGP it is 7.9 (exp. 7.8) and the average for Ala³-H in YTAP is 8.1 (exp. 8.5). If we assume that there are about 20 independent points in the chemical-shift graphs, we can estimate the error in these numbers by dividing the standard deviation by $\sqrt{20}$. The estimated errors amount to: YTGP: 0.07; FTGP: 0.12; YTAP: 0.03 ppm. From this we conclude that the error in the calculated chemical shifts is on the order of 0.1 ppm in all cases. In Fig. 1B the contribution of the ring current to the chemical shift is plotted. In Fig. 1D we have plotted the distance between the proton and the center of the aromatic ring for Tyr¹ and Phe¹, and in Fig. 1C the angle θ between the NH vector and the normal vector \bar{n} on the plane spanned by the CD1, CD2 and CZ atoms. The angle θ is defined such that $\theta = 180^\circ$ corresponds to the NH vector pointing towards the ring. The H-ring distance is almost constant in the YTGP run; in the FTGP run the distance jumps between a low value, corresponding to a low chemical shift, and a high value, corresponding to a high chemical shift. Similarly, θ fluctuates around 120° in the YTGP run whereas it jumps between 120° and 60° in the FTGP run. In Fig. 2 we have plotted the conformation of FTGP after 300 ps and after 1000 ps. The figure clearly shows that an 'open' and a 'closed' conformation exist in our simulation.

There is a good correlation between distance and chemical shift (Fig. 3A), because the largest contribution to the upfield shift of the proton comes from the ring

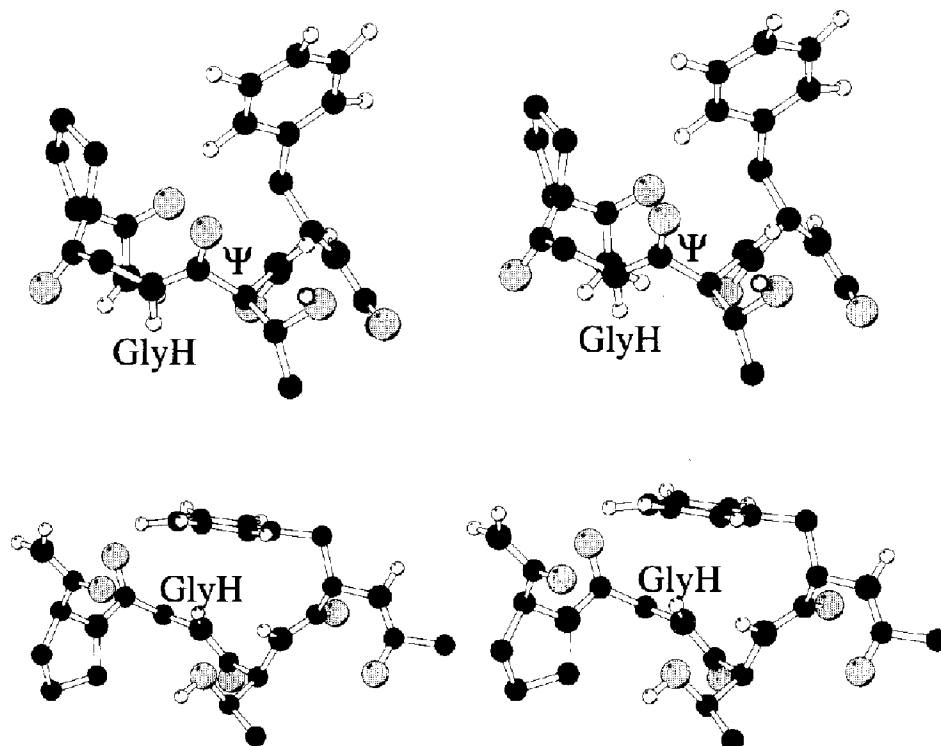


Fig. 2. Stereo plots of FTGP in 'open' (after 300 ps of MD, top) and 'closed' conformation (after 1000 ps, bottom). The conformations are almost identical, except for a rotation around the Thr Ψ angle (indicated). The plots were created using Molscript (Kraulis, 1991).

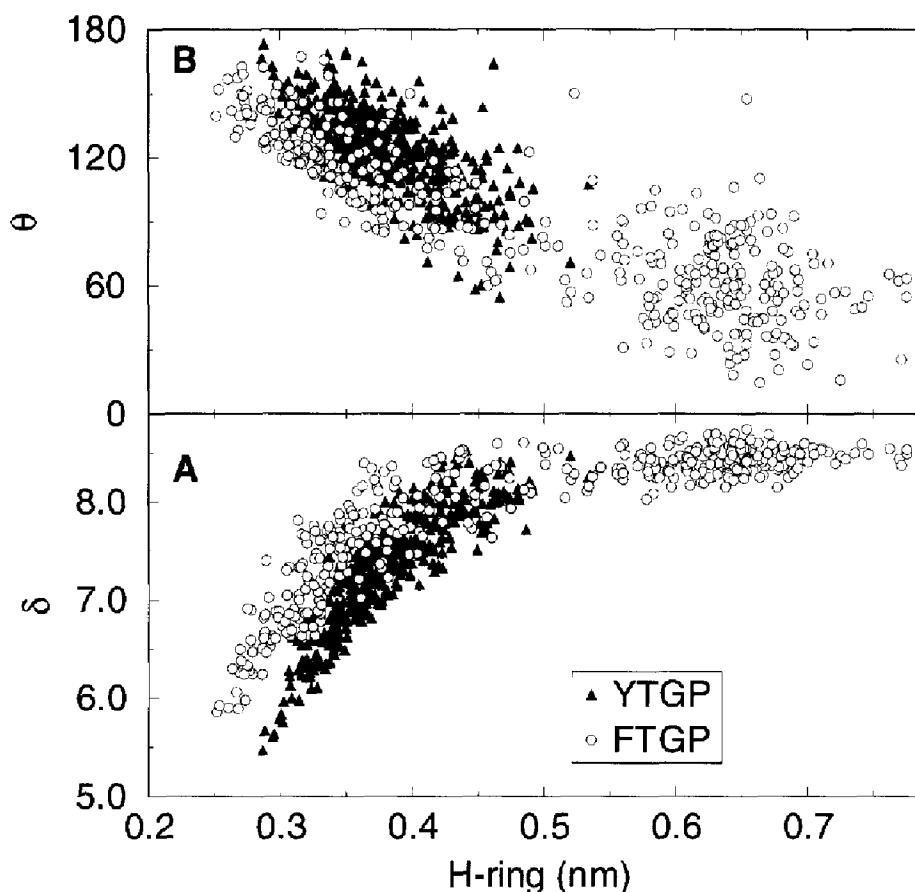


Fig. 3. (A) Correlation between distance and chemical shift for YTGP/FTGP. (B) Correlation between distance and angle for YTGP/FTGP in the G-94 force field with SPC/E water.

currents in the aromatic rings of Tyr¹ / Phe¹. From a distance / angle correlation plot (Fig. 3B) it can be concluded that the average conformation for the YTGP peptide has an NH- \bar{n} angle of 120° and an amide-ring distance of 0.36 nm, which is in good correspondence with MM data (Worth and Wade, 1995), but is somewhat different from the model based on NMR data (Kemink et al., 1993) that suggests an angle of 180°. The FTGP peptide apparently jumps between two distinct but rather well defined conformations, leading to a chemical shift that has an intermediate value when averaged over the whole trajectory. The conformational differences between the two conformations of the peptides can be clarified with a Ramachandran plot (Fig. 4). Here we have plotted the ϕ/ψ angles of the Thr² and the Thr¹¹ residues for the three small peptides and the P₁₋₁₅ peptide, respectively, in the G-94 + SPC/E simulation. It is clear that the YTGP and YTAP peptides both have a single conformation, while the FTGP peptide hops between the two conformations. Since Thr² is in-between Phe¹ and Gly³ in the FTGP peptide, the rotation around its backbone dihedral angles determines whether or not the interaction between Phe¹ and Gly³ can be present. In the P₁₋₁₅ peptide the ψ angle is similar to that in the YTGP peptide, but now the ϕ angle is rotated over -60° to -120°.

Comparison of force fields

In Table 2 the chemical shifts for the Gly³-NH (Gly¹²-NH for P₁₋₁₅) and Ala³-NH protons are listed as the average over the trajectory of each simulation. The agreement between simulation and experiment is remarkable for the simulations of the tetrapeptides in the G-94 force field with SPC/E water. In the same force field with SPC water the experimental data are not reproduced at all, which implies that the role of solvent is critical. The SPC water molecules, which have slightly smaller partial charges than the SPC/E molecules (see Table 1), are able to insert between the aromatic ring and the NH group, making a hydrogen bond. The SPC/E molecules, on the other hand, are more tightly bound to one another because the interaction energy is more favorable due to the higher partial charges. The simulation of the YTGP peptide in the G-93 force field (without hydrogen atoms on the tyrosine ring) also does not produce the correct chemical shift, which implies that these hydrogen atoms are essential for the interaction, and that it is not *only* the solvent that influences the peptide conformation. The simulation of the YTGP peptide using the OPLS force field with TIP3P water does not reproduce the experimental data either.

The hydration of the Gly³-NH group by SPC water can be visualized by plotting the radial distribution func-

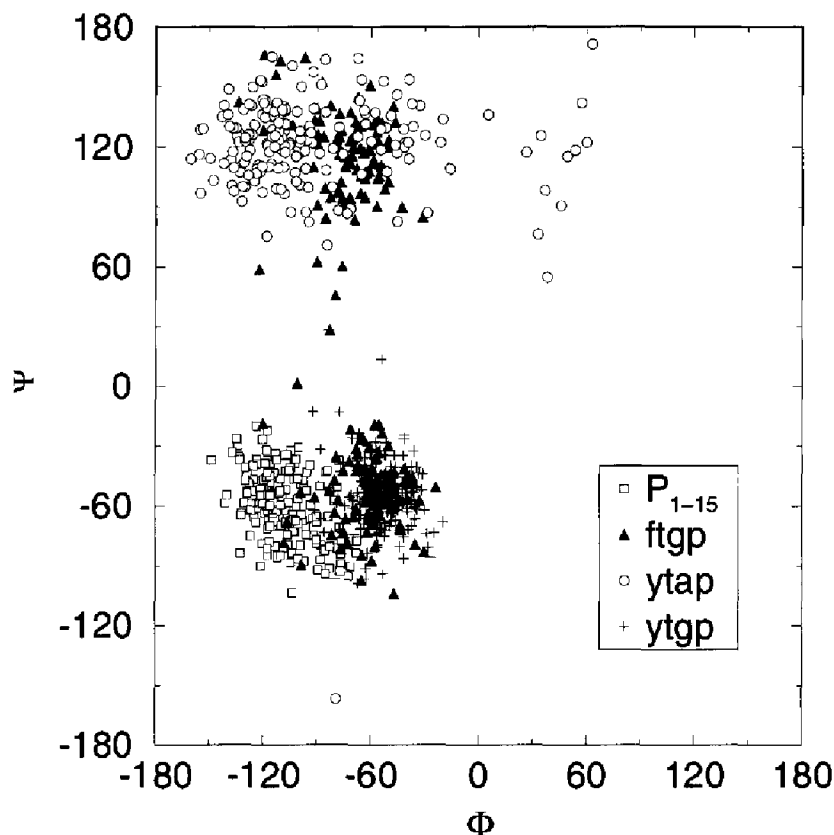


Fig. 4. Ramachandran plot of the Thr residue in all tetrapeptides and the P_{1-15} peptide, simulated with the G-94 force field and SPC/E water.

tion of water oxygen around the N atom (Fig. 5). It can be seen that in the first 250 ps of simulation of YTGP in the G-94 force field the peak is much less pronounced than in the corresponding OPLS simulation, implying no insertion of water molecules between the NH group and the ring. In the last 250 ps of both simulations the differences are much smaller. The RDF has a higher peak and a lower minimum in the OPLS run (750–1000 ps), which implies that water molecules are bound more tightly to the NH group.

In the larger P_{1-15} peptide the interaction between Gly¹²-NH and Tyr¹⁰ is absent most of the time due to a different effect from the solvent. In the SPC/E simulation a hydrogen bond is formed between Thr¹¹-O and Ser¹⁵-H (see Fig. 6A). This hydrogen bond induces the change in

the ϕ dihedral angle of the Thr¹¹ residue mentioned above. Only when this hydrogen bond is absent, the interaction between Tyr¹⁰ and Gly¹² is present (between 500 and 600 ps, see Fig. 6B). In the SPC simulation of the P_{1-15} peptide this hydrogen bond is not formed, because hydrogen bonds with water molecules are favored. Because the amide proton of Gly¹² is also hydrated in this simulation, the average chemical shift is only slightly lower than in the SPC/E simulation.

The influence of solvent on the peptide can also be seen from the secondary structure of the peptide (Fig. 7), which was determined using the DSSP program (Kabsch and Sander, 1983). The α -helix in the P_{1-15} peptide is present a substantially larger fraction of time in the SPC/E simulation than in the SPC simulation. Although this α -

TABLE 2
CALCULATED CHEMICAL SHIFTS δ (PPM) FOR Gly-NH AND Ala-NH PROTONS

Peptide	G-93		G-94			OPLS			Exp.
	SPC	SPC/E	SPC	SPC/E	TIP3P	SPC	SPC/E	TIP3P	
YTGP	7.9	8.3	8.0	7.3	8.0	7.8	8.0	8.1	7.00
FTGP			8.2	7.9	8.3	8.3	8.3	8.4	7.78
YTAP	8.0		8.2	8.1	8.2	8.3	8.2	8.1	8.56
P_{1-15}			7.7	8.0					6.7

The chemical shifts are averaged over a 1.0-ns trajectory for each simulation; experimental values were taken from Kemmink et al. (1993). The errors in the simulation results are 0.1 ppm (see text).

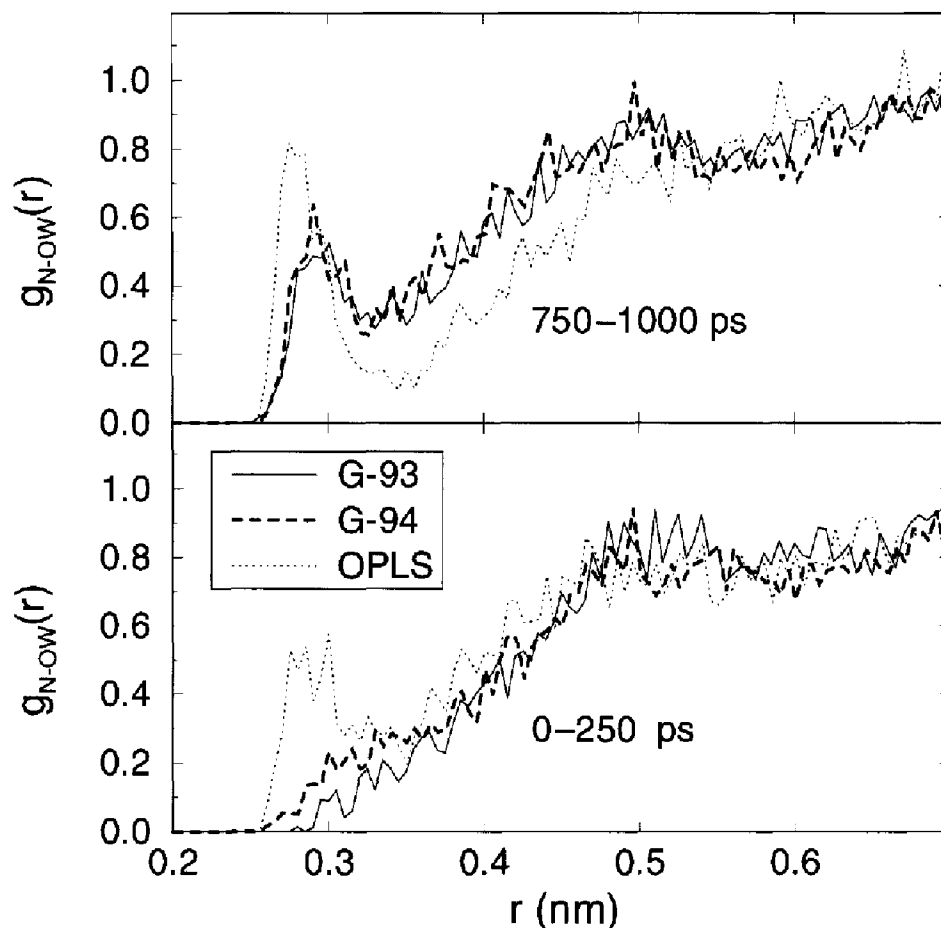


Fig. 5. Radial distribution function of water oxygens around Gly³-N from the YTGP peptide in SPC water for two parts of the trajectory in different force fields.

helix is part of the native BPTI structure, and therefore also present in our starting structure, there is no direct evidence for an α -helical conformation of the first residues of the P₁₋₁₅ peptide in solution. It must be noted that some non-random conformation was detected in the region of residues 3 to 6 by NMR (Kemink et al., 1993).

Discussion and Conclusions

We have performed MD simulations of tetrapeptides in solution and found that it is possible to reproduce NMR data without imposing restraints on the peptides. Moreover, our simulations enhance the understanding of the conformations of these small peptides in solution. The two-state interpretation of the NMR data for YTGP (open \rightleftharpoons closed) is not supported by our simulations. We find a single conformation, in which the aromatic ring is constantly close to the amide proton at an angle of 120°, similar to what was found by MM calculations in the presence of solvent (Worth and Wade, 1995). This conformation allows the proton to interact with solvent simultaneously, but leads to a smaller upfield chemical shift than the perpendicular orientation. In none of our simulations

did we find a hydrogen bond between the Tyr¹-OH group and the Pro⁴-O, in contrast to what was suggested by the NMR data (Kemink et al., 1993). Nevertheless, the possibility of an interaction between Tyr¹ and Pro⁴ cannot be rigorously excluded, as replacing the Pro by an Ala raises the chemical shift of the Gly amide proton by 0.4 ppm (Kemink et al., 1993). Furthermore, it is known from crystal structures that Tyr and Pro residues can make a sort of stacking interaction, although the nature of this interaction is poorly understood (Thornton, 1992). One may be concerned about the length of our simulations, because it is known that peptides containing proline residues equilibrate on a time scale of nanoseconds (Brooks and Case, 1993). However, in YTGP, FTGP and YTAP the Pro residue is the last residue in the sequence, and therefore we think it is not very important for the Tyr-Gly interaction. Nevertheless, it is possible that in a longer simulation the proposed hydrogen bond between Tyr¹-OH and Pro⁴-O would be found. The FTGP peptide, on the other hand, is shown to hop between two conformations in a dynamic equilibrium. Although the number of transitions between the open and closed conformations is limited in the 1.0-ns simulation of

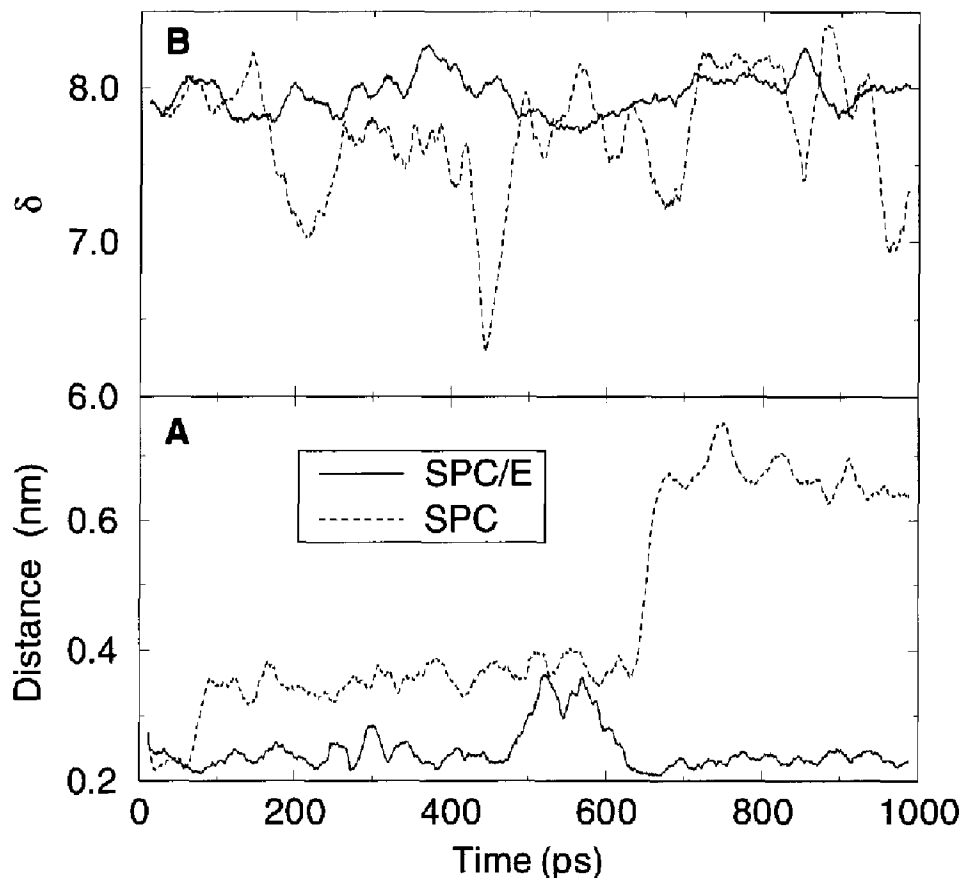


Fig. 6. (A) Hydrogen bond between Thr¹¹-O and Ser¹⁵-H. (B) Chemical shift of Gly¹²-H during simulations of P₁₋₁₅ in SPC/E water and SPC water with the G-94 force field.

FTGP, the resulting average chemical shift is in good agreement with NMR data, and the estimated error of 0.1 ppm is relatively small compared to the difference between the average chemical shifts of the different peptides.

Our results underline that it is necessary to perform simulations that are longer than a few hundred picosec-

onds; to properly sample the conformational equilibrium of the FTGP peptide (Fig. 1) a simulation of 1.0 ns seems to be the bare minimum. It may be possible to improve sampling by running 10 simulations of 100 ps, starting from different conformations (Elofsson and Nilsson, 1993), rather than a single 1-ns simulation, but this is not practical for larger peptides and proteins.

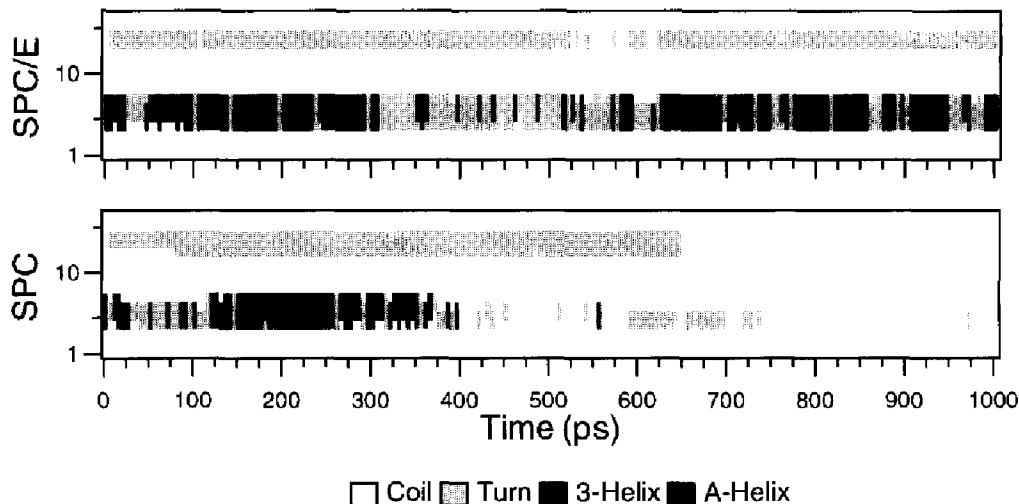


Fig. 7. Secondary structure of P₁₋₁₅ in the G-94 force field using the SPC and SPC/E water models.

The electrostatic interaction between an aromatic ring and an amide proton is intrinsically weak (Burley and Petsko, 1986), but when the amide proton approaches the ring, the π electrons will be polarized and act as a hydrogen acceptor (Cheney et al., 1988). The force fields we have applied do not model polarizability in any way, which means that this particular interaction is not as strong as it should be. On the other hand, when a single SPC water molecule enters the hydrophobic environment of the protein backbone and the aromatic ring, the molecule is overpolarized since the model implies the average polarization of the bulk liquid. For our simulations this means that the interaction between the amide proton and the aromatic ring is too weak, while the interaction between the amide proton and the SPC water molecule is too strong. Thus, the omission of explicit polarizability is the cause of two effects working in the same direction, namely of opening the peptide, which is exactly what we observe. In the SPC/E simulation of the P₁₅ peptide we see that the backbone hydrogen bond between Thr¹¹-O and Ser¹⁵-NH is favored over the amide–aromatic contact. This hydrogen bond *should*, however, be broken in order to allow for the amide–aromatic interaction, but the SPC/E water molecules are not able to break it. It was noted before in simulations of a decane/water monolayer that SPC/E water produces interfaces that are too sharp (Van Buuren et al., 1993), which is similar in nature to what is observed here. The reason that in simulations with the OPLS force field a water is always hydrogen-bonded to the Gly³ amide group (Fig. 5) is probably the higher partial charge on the peptide backbone atoms as compared to G-94. It is possible that the OPLS force field performs better in combination with the TIP4P water model (Jorgensen et al., 1983); we were not able to test this possibility because our software is currently not able to handle virtual sites. It seems that it is not possible to model these slight differences in interaction energy properly without taking polarizability into account. There have been several attempts to include polarizability in small molecules (Ahlström et al., 1989; Caldwell et al., 1990; Cieplak et al., 1990; Åstrand et al., 1991, 1994; Hernández-Cobos et al., 1993; Zhu et al., 1994), but to our knowledge there is no force field for proteins that does employ polarizability. A very promising way to introduce polarizability in molecular dynamics, based on a shell model, is presently under development (Jordan et al., 1995).

Due to the intrinsic weak character of the amide–aromatic interaction it is unlikely that it plays a significant role in protein stability. This notion is confirmed by database searches (Mitchell et al., 1994). The cases we studied here were selected because they are specifically sensitive to details of the force field. It is important to note, however, that hydrophobic interactions are in part electrostatic in nature (Burley and Petsko, 1989) and

therefore the addition of explicit hydrogen atoms to aromatic residues may have an effect on protein stability in MD simulations as well as on protein dynamics (Axelsen et al., 1991; Gordon et al., 1992).

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