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ARTICLE

Molecular dynamics simulations on PGLa using NMR orientational constraints

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Abstract NMR data obtained by solid state NMR from anisotropic samples are used as orientational constraints in molecular dynamics simulations for determining the structure and dynamics of the PGLa peptide within a membrane environment. For the simulation the recently developed molecular dynamics with orientational constraints technique (MDOC) is used. This method introduces orientation dependent pseudo-forces into the COSMOS-NMR force field. Acting during a molecular dynamics simulation these forces drive molecular rotations, re-orientations and folding in such a way that the motional time-averages of the tensorial NMR properties are consistent with the experimentally measured NMR parameters. This MDOC strategy does not depend on the initial choice of atomic coordinates, and is in principle suitable for any flexible and mobile kind of molecule; and it is of course possible to account for flexible parts of peptides or their side-chains. MDOC has been applied to the antimicrobial peptide PGLa and a related dimer model. With these simulations it was possible to reproduce most NMR parameters within the experimental error bounds. The alignment, conformation and order parameters of the membrane-bound molecule and its dimer were directly derived with MDOC from the NMR data. Furthermore, this new approach yielded for the first time the distribution of segmental orientations with respect to the membrane and the order parameter tensors of the dimer systems. It was demonstrated the deuterium splittings measured at the peptide to lipid ratio of 1/50 are consistent with a membrane spanning orientation of the peptide.

Keywords MDOC · Molecular dynamics simulations · Orientational NMR constraints · ^{2}H NMR · Oriented samples · PGLa peptide · Order parameters · Force field calculations · Cell penetrating peptide

Introduction

Using anisotropic media, such as partially oriented bicelles or macroscopically oriented membranes, NMR investigations can reveal a wealth of information about molecular properties, namely conformation, orientation and dynamics. This information is not easily extracted from spectra and methods of computational NMR are needed to analyse, interpret and confirm the data. A proper way to account for motional averaging of the NMR parameters caused by molecular mobility is to run all-atom MD simulations. When applied to bio-membranes, however, such simulations pose some problems: (1) large size of the system, and related to that (2) long simulation times as well as (3) a dependency on the type of force field. This last point is important since the force field—especially its parameterization—has to be optimally designed for water, lipids and the guest peptide. The first two problems occur since the water environment of the lipid bilayer has to be included into the MD simulations. To avoid boundary effects the volume of interest has to be surrounded by multiple copies of the central cell (this is automatically performed by periodic boundary conditions). The NMR time scale starts at nano seconds but membrane reorganizations will

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proceed in much longer time spans and the MD simulation should cover these long times spans. Nevertheless, such simulations have been demonstrated to be feasible for moderately sized membrane segments hosting medium size molecules (Tieleman et al. 2001). Ulmschneider et al. (2012) performed such MD simulations for PGLa in lipid bilayers with two force fields (CHARMM runs of 1 μs and OPLS runs of 2 μs). These simulations revealed a significant dependence of the results on the choice of the force field. The predicted dimer of PGLa was found only in the CHARMM simulations. To validate their results the authors (Ulmschneider et al. 2012) used 11 deuterium splittings measured on Ala-d_3 labeled PGLa samples. To calculate the residual quadrupolar splittings the authors placed perfect α-helices on the PGLa positions obtained in the MD simulation. The RMS deviation of calculated and experimental values turned out to be always larger than 3 kHz; compared to the experimental error of 1 kHz (Strandberg et al. 2006) there seems to be need for further investigations.

Here, we apply a new MD method to deduce structural and dynamics information on the antimicrobial peptide PGLa and its dimer. PGLa is a 21-residue cationic peptide (GMASKAGAIAGKIAKVALKAL-NH_2) from the magainin family of peptide antibiotics present in frog skin, which folds into an amphiphilic α-helix when bound to lipid bilayers.

With our alternative strategy (Sternberg et al. 2007) the oriented medium can be avoided due to the presence of orientation dependent pseudo-forces that are directly derived from measured NMR parameters. Therefore, the method is called molecular dynamics with orientational constraints: MDOC. Since these orientational pseudo-forces propel molecular motions and reorientations, MDOC can be considered in some respect as an accelerated molecular dynamics approach. Due to this property motionally averaged parameters as observed in oriented NMR experiments are calculate directly on the MDOC run. As a result, the full information about molecular orientation, order, segmental motions and even aspects of the molecular conformation can be revealed.

Experimentally observed NMR values usually represent a time and ensemble average over the motions of molecules and knowledge of the geometry and the nature of the motions are prerequisite for properly interpreting NMR tensors. Even for a completely stiff molecule five components of an order tensor are needed to describe the motion requiring five NMR measurements for its evaluation. In the simplest case of isotropic motions only one order parameter is needed and these order parameters or order tensors are not known a priori. There is a remarkable advantage of MDOC simulations involving orientational constraints: in contrast to the traditional approach the molecular motions are intrinsically included and total or segmental order tensors can be extracted from the MDOC trajectories.

In a first application on PGLa (Sternberg et al. 2007) we used as constraints only the deuterium splittings of the helical part of the molecule (peptide to lipid ratio of 1:200): for the other three alanine sites of PGLa the splittings were predicted. Later on two of the predicted values were measured (Strandberg et al. 2009) and Table 1 gives a comparison of simulated and new experimental data. The two values of Table 1 are determined for the more flexible end of the peptide chain and the predictions deviate by around 3 kHz from the experiment. This deviation comes from the fact that the motion of the flexible ends of the helix was not constraint by experimental values.

In the present study we not only included the flexible ends of the molecule but investigated the possibility of dimer formation. From the results of measurements at higher peptide to lipid ratios (1:50) it is known that the lipid changes dramatically its behaviour and its orientation with respect to the membrane (see Fig. 1). Therefore, we performed MDOC simulation with a PGLa dimer to investigate its stability and orientation. In such a MDOC simulation both molecules of the dimeric complex simultaneously have to fulfil the experimental constraints.

One aspect of antimicrobial peptides like PGLa is their ability to penetrate bio-membranes. Some of these so called cell penetrating peptides (CPP's) induce pores in lipid bilayers by accumulating on the membrane surface and interacting with the lipids. On the membrane surface the PGLa gathers in a α-helical state displaying a hydrophobic and a hydrophilic face (see Fig. 2). The surface bound state and its concentration dependence was investigated in some NMR studies, see e.g. (Tremouilhac et al. 2006). The process and the pore formation was studied for a peptide of the magainine family by Leontiadou et al. (2006) who simulated its interaction with lipid membranes using molecular dynamics. In MDOC simulations the membrane is in principle substituted by orientational constraints and therefore pore formation cannot be

Table 1 Predicted (Sternberg et al. 2007) and measured ^{2}H splittings of PGLa at a peptide to lipid ratio of 1:200

Site	Predicted ^{2}H splitting (kHz)	Measured ^{2}H splitting[a] (kHz)
Ala 17 CD_3	+17.1	14.0
Ala 20 CD_3	−25.5	28.3

[a] No signs from original experiments (Strandberg et al. 2009)

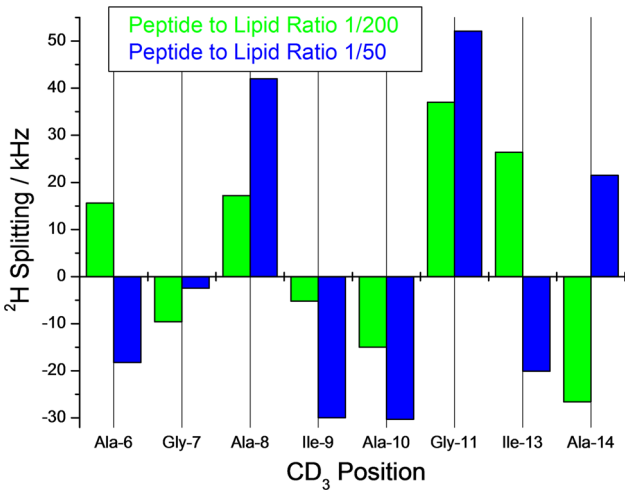

Fig. 1 Change of the ^{2}H splittings with increasing the peptide to lipid ratio from 1/200 to 1/50, data from Tremouilhac et al. (2006)

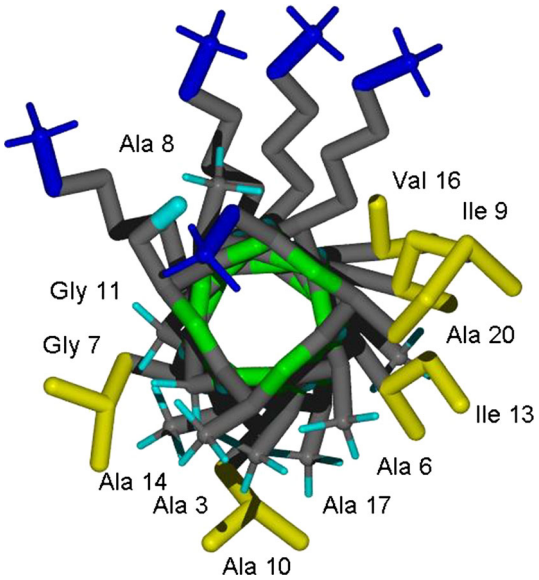

Fig. 2 View along the long axis of PGLa modelled as an idealized α-helix. The quadrupolar coupling tensors of the 12 ^{2}H labelled groups were included into the MD simulation as orientational constraints. Positive charged groups are presented in *blue* and hydrophobic groups in *yellow*

directly observed. In the process of penetrating the membrane through a pore PGLa will undergo a transition from the surface orientation on the membrane to a membrane spanning state; and this change of orientation leaves its fingerprints in oriented NMR spectra. We can use the MDOC simulations to investigate whether a membrane spanning orientation could be accomplished without violating the NMR constraints.

Some membrane active peptides like e.g. Gramicidine A form ion channels and represent a stable membrane spanning structure. MDOC simulations were used to investigate the oriented motions of the Gramicidine A channel and the orientation of the side chains of the tryptophans (Sternberg et al. 2009).

Materials and methods

Force field calculations and orientational pseudo-forces

The MDOC simulations are performed using the COSMOS-NMR force field, which has been applied in a number of previous structure investigations involving distance and chemical shift constraints (Sternberg et al. 2004; Witter et al. 2002, 2006), and for the force field (Sternberg et al. 2001; Witter et al. 2015). Previously, orientational NMR constraints have been introduced into COSMOS-NMR and demonstrated on three examples including PGLa to produce a detailed picture of the molecular motions and orientations in oriented membranes (Sternberg et al. 2007). A freely available multiplatform COSMOS-Backend (C++) version was compiled for several operation systems including Windows and Linux.

To include experimental constraints into MD simulations, pseudo-energy terms are added to the molecular energy provided by the force field. These pseudo-energies are defined as functions of the difference between the experimental and a calculated tensor property, $\boldsymbol{P}^{\boldsymbol{i}}$:

$$E_{pseudo} = \frac{k}{2}\sum_{\alpha\beta}\sum_{i}\left(P_{\alpha\beta}^{theo,i} - P_{\alpha\beta}^{\exp,i}\right)^2, \quad (1)$$

where k is a force constant which is chosen to adjust the size and unit of the energy. The first sum runs over all observed tensor components but does not need to include all possible elements. From a 1D NMR measurement on a single label in an oriented or monocrystalline sample, only the zz-component of the related tensor can be experimentally determined. However, any additional tensor component included as constraint into the calculations will improve the characterization of the orientation of the system. Due to the nature of the tensor transformation each principal axis of the tensor can be inverted without changing the observed NMR frequency. Therefore, eight discrete tensor orientations are equivalent (+1+1+1, −1+1+1, +1−1+1,…, −1−1−1), even if all elements of the observed tensor are used as constraints. If, however, only the three principal values of the tensor are known, then in addition to this eight-fold degeneracy a continuous set of orientations (instead of discrete possibilities) will be compatible with the experimental data from a single label. Therefore, a larger number of orientational constraints from several labels are necessary to determine the orientation of a rigid molecular segment in an unambiguous

way. Obviously, if a molecule is intrinsically flexible, an even higher number of constraints will be required.

For the calculation of the time average of the quadrupolar coupling tensor an exponential memory function had been introduced. The differences between calculated and experimental values become time dependent and ensure a well defined time scale for mean alternations of the pseudo-forces. This time scale represents the life time of an orientation of the molecule or a mobile segment. The memory time constant—in this case 200 ps—leads to a time dependence of the pseudo-forces and is used to control molecular reorientations.

To include the NMR constraints into the equations of motion, pseudo-forces have to be calculated from the respective pseudo-energies (Eq. 1):

$$F^{A}_{x_{\gamma_j}} = k\ \ s(\Delta P) \sum_{\alpha\beta}^{3} (P^{theo_A}_{\alpha\beta} - P^{\exp_A}_{\alpha\beta}) \frac{\partial}{\partial x_{\gamma_j}} P^{theo_A}_{\alpha\beta}$$
$$\frac{\partial}{\partial x_{\gamma_j}} P^{theo_A}_{\alpha\beta} = \left(T_{\beta\beta'} \frac{\partial}{\partial x_{\gamma_j}} T_{\alpha\alpha'} + T_{\alpha\alpha'} \frac{\partial}{\partial x_{\gamma_j}} T_{\beta\beta'} \right) P^{theo_A}_{\alpha\beta} \quad (2)$$

They are obtained as the derivatives of the energies with respect to the coordinates of the atoms x_γ. In the case of orientational pseudo-forces we have to derive the transformation matrices $\boldsymbol{T}$ [see Eq. (3), (4)] with respect to the coordinates of the atoms that were used in their definition (Sternberg et al. 2007). In Eq. (2) k is a force constant to adjust the strength and the dimension of the forces and $s(\Delta P)$ is a scaling factor introducing a potential dependency on a width parameter ΔP which should be in the order of the experimental error. This potential ensures that the calculated splittings (tensor properties) vary within the MD trajectory in the bounds of ΔP.

The integration of the equations of motions is based on Verleťs algorithm (1967), and time steps of 0.5 fs were employed to sample all high frequency hydrogen atom vibrations.

In constrained MD simulations it is generally necessary to control the temperature. This is accomplished by coupling the molecular system to a heat bath which dissipates the heat generated by the pseudo-forces. All prevailing differences between the constraints and their calculated values are sources of heat. To obtain a NTV assemble (with conserved particle number N, temperature T, and volume V), we introduced a proper thermostating procedure (Evans and Morris 1990). The coupling to the thermostat is controlled by a coupling time constant η which should be much larger than the time step. This time span η allows an adjustment of the range of thermal fluctuations in the simulated molecular system (Table 2).

To prevent too large pseudo-forces at the start of the MDOC simulation the pseudo-forces were gradually increased towards their final values with a time dependent scaling factor $f = 1 - e^{-t/\rho}$ which approaches the value 1.0 in an exponential fashion. The time constant was set in most cases to 200 ps (the order of the memory time) leading to a relatively smooth course of the temperature.

Table 2 General parameters for the MD simulations with orientational constraints

Parameter	Value
Target temperature	290 K
MD time step	0.5 fs
Coupling time η to the heat bath	0.01 ps
Pseudo-force width ΔP	1 kHz
Memory decay time τ for the property average	200 ps
Time constant ρ for the exponential rise of pseudo-forces	200 ps
Total MD duration	1 ns

From the NMR studies it is known that PGLa performs a rapid rotational diffusion (on the NMR time scale) about the membrane normal. This motion provides a second possibility to scale the pseudo-forces properly by calculating rotational averages of the quadrupolar tensors during every MD step. In order to obtain an averaged tensor three tensors values are averaged that are rotated in 120° steps around the director axis (in this case the membrane normal oriented parallel to the B_0 field direction). All off-diagonal elements of the calculated tensors within the laboratory system become zero and finally only the principal values of the tensors are left as constraints.

When applying NMR orientational constraints during a MDOC run the resulting pseudo-forces will "heat up" the system by exciting its rotational degrees of freedom and some net rotational motion will prevail up to the end of the simulation. In common MD simulations any overall molecular rotation and translation is eliminated from the velocity steps, since these external degrees of freedom are not of interest. In the present orientationally constrained calculations, however, only the net translations of the systems are removed.

In our investigations two types of orientational constraints are introduced: (1) deuterium couplings from $-CD_3$ groups of deuterated alanins and (2) couplings of $^2H_\alpha$ atoms of glycines. For the static value of the $-CD_3$ group we used a coupling constant of $C^Q = 58.33$ kHz (Sternberg et al. 2007). The orientational constraints are assigned in all cases on C_α–C_β bonds and the pseudo forces act on these bonds. For some of the NMR measurements (Tremouilhac et al. 2006) PGLa derivatives are synthesized replacing amino acids like isoleucines and glycines by deuterated alanins. In the simulations these substitutions are avoided and the structure of the peptide was kept in its original state. The orientational constraints acted in the

case of isoleucines on C_α–C_β segments or on the appropriate C–H_α bonds of glycine.

Model building for the PGla monomer and dimer

It is known from previous NMR studies (Bechinger et al. 1998) that membrane-bound PGLa forms a α-helical conformation between residues 6 and 21. Therefore, the MDOC simulation was started with an idealized α-helix, and to keep the molecular model helical, 18 soft distance constraints of 1.86 Å were introduced to enhance the backbone hydrogen bonds. The first hydrogen bridge constraint was placed between the carbonyl oxygen of lysine 5 and HN of isoleucine 9. In this O(n) to H(n + 4) manner we proceeded to alanine 17. One additional hydrogen bridge constraint was introduced (2.1 Å) between the carbonyl oxygen of lysine 19 and one of the amid protons of leucine 21 giving a total number of 18 distance constraints per molecule. PGLa is helical only in contact with the membrane—so called "conformative selection"—and therefore it is necessary to preserve the helical state by proper soft constraints.

It also was taken into account that the four lysine side chains and the N-terminus are positively charged. Since charged NH_3^+ groups have a strong tendency to form hydrogen bonds, we added a water molecule near each hydrogen atom of a charged group. In this way undesired hydrogen bonds of lysines to the backbone could be prevented, and indeed at the end of the simulation all 15 water molecules were still in contact with the peptide.

For the PGLa dimer two monomers where placed antiparallel side by side with a tilt of 99° obtained from the simulation related to low peptide to lipid ratio of 1:200. The two ideal helices were oriented with their hydrophobic faces opposite to each other. Water molecules were added in the same way to the model as in the case of the monomer (the model building was performed with COSMOS; Sternberg et al. 2001).

For both models together the partial atomic charges were calculated (Sternberg et al. 2001; Sternberg and Möllhoff 2001; Witter et al. 2015) on the BPT-6-31G(d, p) level taking into account charged groups and water molecules. This charge distribution is used to calculate non-bonded energies in COSMOS-NMR force field simulations.

At a peptide-to-lipid ratio of 1:50 in DMPC at 35° C it was found that the peptide undergoes fast rotational diffusion about the membrane normal. From the deuterium splitting data (Strandberg et al. 2009) we could derive the full quadrupolar coupling tensors for the 12 and in the case of the dimer 24 ^{2}H-labelled sites and used these as orientational constraints for the MDOC simulations. From the paper of Strandberg et al. (2006) we obtained the signs of 8 ^{2}H splittings which were derived from a comparison to fluorine NMR experiments. To obtain the signs of the quadrupolar couplings for the newly measured three sites (3, 16, 17 and 20) we performed preliminary 1 ns MDOC simulations of the monomer by taking only the former 8/16 tensors as orientational constraints into account and calculated the MD mean value of the other 4/8 coupling tensors. For the dimer a preliminary MDOC simulation was also performed omitting the constraints at Val 16, Ala 17 and Ala 20, as presented in Table 3. Due to the flexibility of the peptide ends it is difficult to make precise predictions concerning related deuterium couplings. However, the signs could be determined for the final simulations. The final simulations were performed inculding all constraints and the signs from the preliminary MDOC simulations.

Results and discussion

The PGLa monomer

After performing a 1 ns MDOC simulation the mean temperature of the monomer was 1.1 K above the target temperature of 290 K. This weak temperature increase indicates that only small pseudo-forces contributions prevailed. The final RMSD of the backbone hydrogen bond lengths from their ideal values was only 0.3 Å, thus confirming that the helix of central part of the molecule is only minimal distorted during the MDOC simulation.

With the constraint-driven MDOC simulations it was possible to reproduce the experimental ^{2}H-NMR data, compiled in Table 3. All calculated frequencies are well within the experimental error margins [as estimated from the experimental line widths (Strandberg et al. 2006)].

The orientation of the molecule fluctuated in a wide range of values. In Fig. 3 the trajectory of the tilt angle θ (angle of the membrane normal with long axis of the molecule) is shown. The long axis was defined as the axis a of least inertia. From this trajectory it is obvious that the molecule performs large jumps between two distinct states.

The PGLa dimer

As in the case of the monomer both molecules fulfilled most orientational constraints within an experimental error of 1.0 kHz. The largest deviation from experiment with 2.1 kHz is found in the case of Ile 9. Generally, the deviations from the isoleucine sites are larger because the measurements are obtained from derivates where the isoleucines are replaced by deuterated alanines. The mean temperature was 2.2 K above the target temperature indicating that only very week pseudo-forces influenced the structure and motion of the two molecules. The helical structure was preserved due to the distance constraints that

Table 3 Calculated deuterium quadrupolar splittings and order parameters from MDOC simulations of PGLa, compared with the experimental ^{2}H-NMR data in DMPC at a peptide to lipid ratio 1:50

Site	MDOC Monomer 12 constraints (kHz)	MDOC Dimer 9 constraints (kHz)	MDOC Dimer 12 constraints (kHz)	NMR experiment (kHz)[b]
Ala 3	+26.2	+26.1, +25.7	+26.2, +26.1	26.1[c]
Ala 6	−18.5	−17.3, −17.5	−18.6, −18.8	−18.2
Gly 7	−2.4	−2.3, −2.5	−2.5, −2.4	−2.5
Ala 8	+42.1	+41.6, +41.5	+41.6, +42.2	+42.0
Ile 9	−29.2	−28.1, −27.4	−29.5, −27.9	−30.0
Ala 10	−30.1	−30.1, −29.9	−30.4, −30.9	−30.3
Gly 11	51.7	52.6, 52.1	50.4, 51.3	52.1
Ile 13	−20.3	−19.0, −20	−21.4, −21.0	−20.1
Ala 14	−21.4	−22.1, −22.1	−21.3, −22.0	−21.5
Val 16	30.6	(14)[a]	+30.1, +30.3	30.6
Ala 17	−25.7	(−9.7)[a]	−24.9, −25.3	25.5
Ala 20	26.8	(4.7)[a]	+26.6, +26.3	27.3
S_{aa}	0.16	−0.42, −0.44	0.70, 0.65	
Order S	0.17	0.95, 0.97	0.72, 0.83	
Biaxiality	−0.03	0.02, 0.01	−0.05, −0.05	

[a] Predicted values to obtain the sign of the splitting for the simulation with 12 constraints

[b] Experimental values taken from Strandberg et al. (2009). The error of these measurements is estimated to be in the range of 1 kHz (Strandberg et al. 2006)

[c] Private communication (Afonin 2010)

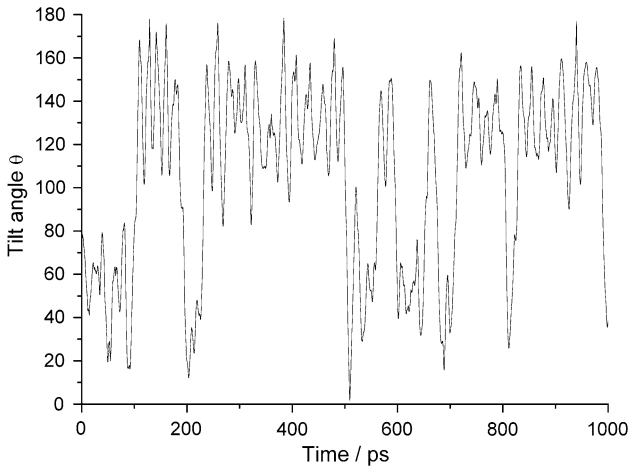

Fig. 3 Trajectory of the time development of the angle θ of the long axis of the monomeric PGLa molecule with the membrane normal (peptide to lipid ratio 1/50). The long axis is defined as the axis *a* of least inertia. The molecule fluctuates between two orientations of around 40° and 140° (the mean value between these two states is $\theta \sim 105°$)

deviated by 0.23 Å (RMSD value) from the ideal hydrogen bridge lengths. This helical behaviour is in accordance with oriented circular dicroism (OCD) measurements (Bürck et al. 2008).

It is remarkable that the two molecules formed a stable dimer within first 50 ps that kept together for the whole remaining duration of the MDOC simulation. Due to the fact that pseudo-forces are gradually switched on within the 200 ps, the system was at the start of the MDOC simulation hardly influenced by pseudo-forces and the dimerization occurred spontaneous (see Fig. 4). Within the following 50 ps the dimer changed its tilt dramatically and the final values were near 23° and 167° (Fig. 6). The helical part from glycine 1 to lysine 5 un-winded to form a sharply kinked L-shaped structure. This result was in strong contrast to our preliminary simulation (for obtaining signs) with nine constraints: the dimer was formed in a similar manner but the orientation was not far from the initial surface state. What we observe here is an example of a delicate interplay between structure and orientation. The non-helical ends of the molecule that cannot be stabilized by intra helical hydrogen bonds are essential for the formation of the dimer. When the orientation of these ends is controlled by constraints as in the case of Ala 3 and Ile 13 to Val 16 we obtain different results for the orientation of the whole molecule. This can be easily seen from the different order tensor components of the long axis of the molecule S_{aa} in the simulation with 9 or 12 constraints (see Table 3). Table 3 lists the components of the molecular Saupe tensor, which are used to describe the average alignment of a molecular fixed frame (i.e. the principal axes of inertia) with respect to the laboratory frame. The Saupe order tensors are calculated in analogy to Eqs. (3) and (4) only that the principal axes of the tensor of inertia are used to construct the rotation matrices $\boldsymbol{T}$. However, this

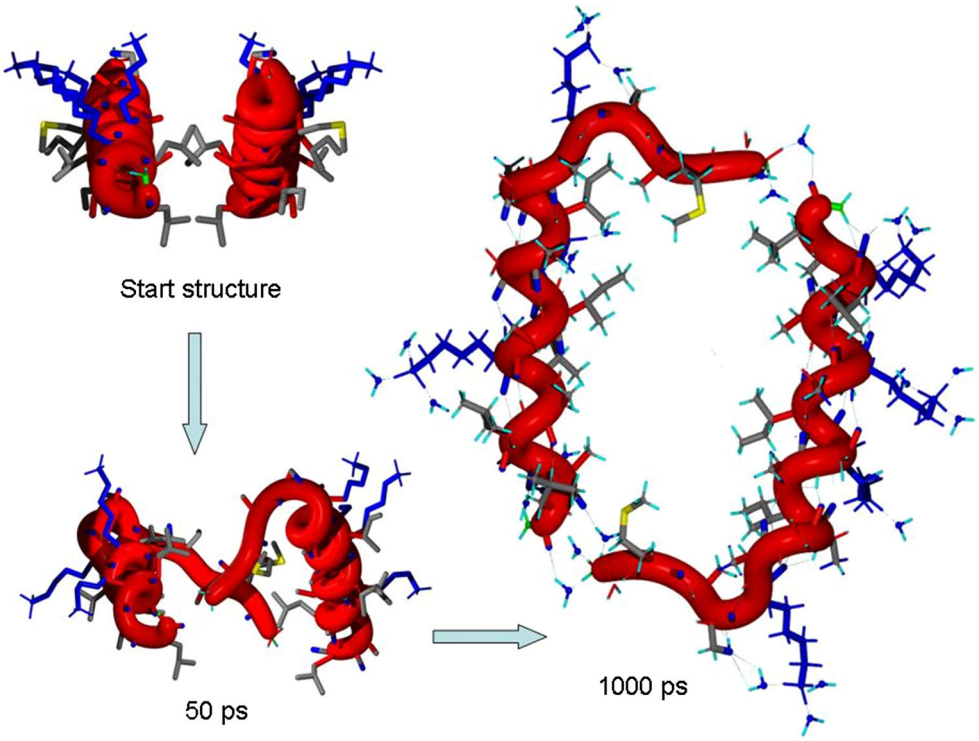

Fig. 4 Formation of the PGLa dimer in a MDOC simulation. The magnetic field is aligned upwards within the paper plane. After 50 ps of simulation the dimer structure on the right side is formed and persists till the end of the MDOC simulation

Saupe tensor does not represent fast reorientation of the molecule around the membrane normal because we introduced an independent averaging for that as described above: no pseudo-forces were driving this kind of motion in the simulation, since a rotational mean value was introduced. Here, the order tensor represents only the wobble of the long *a*-axis and the pronounced oscillations about this molecular axis (see Figs. 4, 6). As expected from the distribution of polar and hydrophobic residues on the different faces of the helix, it is seen that the Saupe tensor is not diagonal. The components S_{aa} along the long molecular axes indicate quite different mean orientations for the monomer and dimer simulations. After diagonalization of the order tensor (see Table 3) we obtain its principal values and can define an order parameter S as well as a biaxiality parameter ξ: $\xi = 0.05$, $S = 0.72$ and 0.83 which are not far from the values that were obtained in a previous investigation at lipid to peptide ratio of 200 (Sternberg et al. 2007).

Calculating all splittings from the single final orientation of the PGLa dimer simulation we would get a RMS deviation as large as 13 kHz between theoretical as experimental values. These splitting differences could not simply be diminished by scaling down the theoretical values by a scalar molecular order parameter since we have large maximum negative and positive deviations: not only the statistical tumbling of the molecule but also backbone and side chain motions are responsible for the order tensors and hence the observed splittings. From the time development of the orientation of the principal axes systems of the quadrupolar interaction tensors it is possible to calculate local order tensors for every deuterated site (Sternberg et al. 2007):

$$\begin{aligned} P^{A_t}_{\alpha\beta} &= T^{PAS_A}_{\alpha\alpha'} T^{PAS_A}_{\beta\beta'} P^{<>^t}_{\alpha'\beta'} \\ W^{A}_{\alpha\beta} &= \frac{1}{2}\left\langle 3T^{PAS_A}_{z\alpha} T^{PAS_A}_{z\beta} - \delta_{\alpha\beta} \right\rangle_t \end{aligned} \tag{3}$$

The property tensor $\boldsymbol{P}^A$—in this case the quadrupolar splitting tensor for the site A—is transformed from its principal axes system using the transformation matrix denoted by $\boldsymbol{T}^{PAS}$ and from the time average—denoted by $<>_t$—of these matrices the local order tensors $\boldsymbol{W}^A$ are calculated. The transformation matrices $\boldsymbol{T}^{PAS}$ are computed from the unit vectors of the principal axis systems:

$$T^{PAS} = (\vec{e}_1\ \vec{e}_2\ \vec{e}_3) \tag{4}$$

Since we assigned deuterium CD_3 splittings to C_β–C_α bonds (and in the case of glycine to a H_α–C_α bond) the local order tensors of Eq. 3 represent the behaviour of these bonds.

The local order tensors are diagonalized to obtain order parameters S and the biaxiality values. The local biaxialities are not negligible and their ratios range from 0.009 to 0.15. From Fig. 5 it is obvious that the local order is far from uniform and as expected the order parameters are lower at the ends of the molecules. This analysis provides

strong arguments that peptides like PGLa cannot generally analysed using stiff molecular models. In the case of the constrained MD simulation with splittings obtained at the low peptide to lipid ratio of 1:200 (Sternberg et al. 2007) we obtained molecular orientations that were comparable to the data obtained by (Strandberg et al. 2006) using a static RMS analysis. However, for the data discussed in this paper this does not hold anymore. The static RMS analysis can probably readily be used for stiff systems—as for instance the cholesterol backbone—but for flexible peptides related results become doubtful.

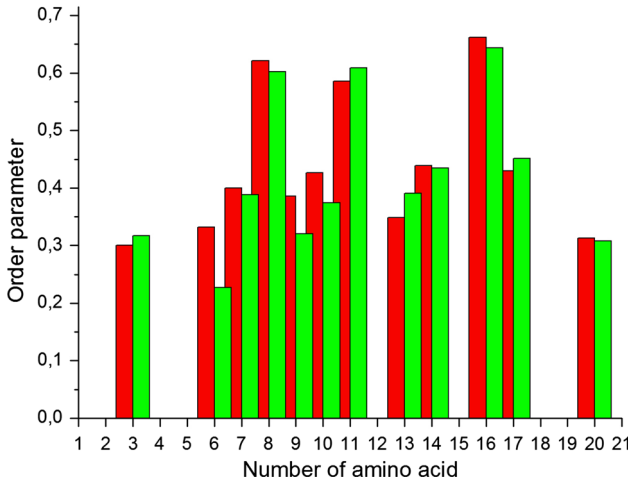

Fig. 5 Local order parameters (absolute values) calculated from a MD trajectory of the orientation of the quadrupolar splitting tensors of the PGLa dimer. The order parameters of chain A are shown in *green* and that of chain B in *red*

The principal axes of inertia offer a suitable molecule-fixed frame of reference to describe the peptide orientation. In the PGLa the *a*-axis of least inertia points along the helix, and the *c*-axis of highest inertia is located between the hydrophilic lysine-rich face and the hydrophobic face of the molecule formed by alanines, valines, leucines and isoleucines. Figure 6 depicts the time development of the tilt angle θ of the long axis of the molecules with the membrane normal (in our case also the z direction of the B_0 filed) and the right panel displays the distribution of this angle. The long axis in our calculations is defined as the axis of least inertia a. As can be seen from this figure the long axis oscillates by $\pm 10°$ around the mean tilt of 23°. It should be kept in mind that the molecules additionally to this wobble rotate fast (with respect to the NMR time scale) around the membrane normal.

The outcome of the MDOC simulation is most appropriately illustrated in Fig. 7, which visualizes the alignment and dynamic behaviour of the PGLa dimer in DMPC. Here, the peptide orientations during the MD simulation are presented as a scatter plot of the inertia axes a, b and c on the surface of a unit sphere. A narrow range of orientations is seen for the a-axis representing the peptide helix (see also Fig. 4). The time-averaged tilt angle of the a-axis with respect to the membrane normal for the last 800 ps is found to be 23° and 157°, with a mean deviation of about ±4°. In fact both molecules move like it is expected for a tightly bound dimer.

In nature, heat is transferred by stochastic interactions with other molecules, thereby introducing a stochastic behaviour of molecular rotations and re-orientations. This stochastic aspect is not present in our MDOC simulations,

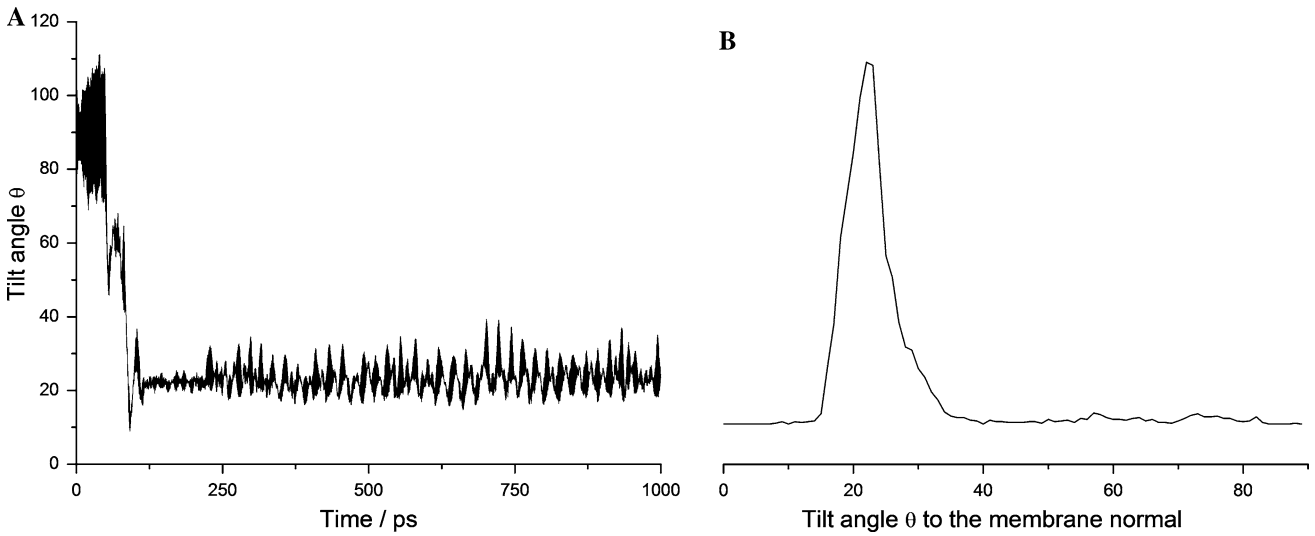

Fig. 6 **a** MDOC simulation of an anti-parallel PGLa dimer. Trajectory of the angle θ of two long axis of the PGLa molecules (axis of least inertia a) with the membrane normal. The tilt angle of the second molecule is displayed as $180 - \theta$. **b** Distribution of the angle θ calculated from the trajectories of axis a. The most abundant angel is 23° tilted from the membrane normal

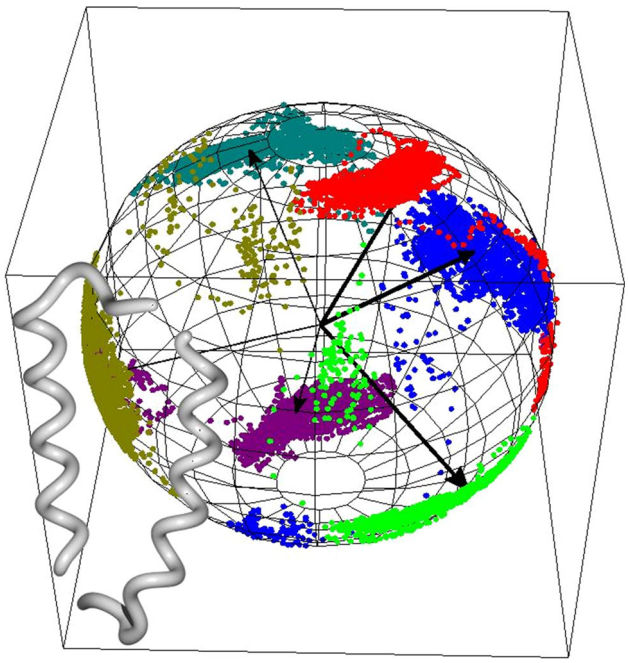

Fig. 7 Visualization of the alignment and motional behaviour of the dimer of the α-helical peptide PGLa (only the backbone is shown) in a DMPC membrane. From a 1 ns MDOC simulation using experimental ^{2}H-NMR constraints the instantaneous orientation of the three axes of inertia *a*, *b* and *c* (the axis of the first molecule are bold and the axis of the second molecule fine) are displayed as a scatter plot on a sphere, representing pico-second-snapshots of these axes orientations. The scatter of the *a* axes is displayed in *red* and *magenta*. The *b* axes pointing into the direction of the lysine side chains is shown in *green* and *dark green*. The poles of the sphere are oriented parallel to the membrane normal. The helix long axes display only a small scatter with a mean tilt angle of 23° and 157°

but nonetheless the amplitudes, velocities and directions of the motions adopt realistic values as the NMR constraints have to be satisfied.

From the point of view of the NMR data there is no direct evidence for the PGLa dimer formation. When going from the low lipid concentration (peptide to lipid ratio of 1:200) to higher lipid concentrations (peptide to lipid of 1:50) as investigated in this paper a clear change of the ^{2}H ($-CD_3$) and ^{19}F ($-CF_3$) splittings was observed (Strandberg et al. 2006). This change was attributed to a dimer formation. In our MD simulation an anti-parallel dimer forms after 50 ps (see Fig. 4). To the dimer model three water molecules were added to all charged $-NH_3$ groups. It turned out that the water molecules form strong hydrogen bonds and none is lost during the MDOC simulations. The three water molecules at the terminal $-NH_3$ groups form hydrogen bonds to the carbonyle oxygens of alanine 20 and leucine 21 of the neighbour molecule. Beside the hydrogen bonds a further cogent contribution to the PGLa dimer formation obviously comes from sulphur atoms in methionine 2 which are opposite to $-CH_3$ groups of leucine 21.

In analogy to our predicted PGLa dimer also magainin-2 forms in membranes such kind of structure that was analysed by NMR (Wakamatsu et al. 2002). The magainin-2 dimer is also a head to tail dimer as in our case of the predicted PGLa dimer but the molecular contact is mediated by tryptophane rings (see Fig. 8).

Conclusions

NMR in oriented media is a valuable technique to gain insight into the behaviour of peptides and proteins. If tensorial properties like quadrupolar or dipolar splittings are measured the data have to be interpreted in terms of orientational order and dynamics. In a previous paper (Sternberg et al. 2007) we developed a new method called MDOC in which all-atom MD simulations combined with NMR data obtained from oriented samples were used to study the motional tensor averages. For this a molecular mechanics force field, in this case COSMOS-NMR (Sternberg et al. 2001; Witter et al. 2002; Witter et al. 2015) was extended to include pseudo-forces, which drive the molecular dynamics to meet the NMR constraints. They

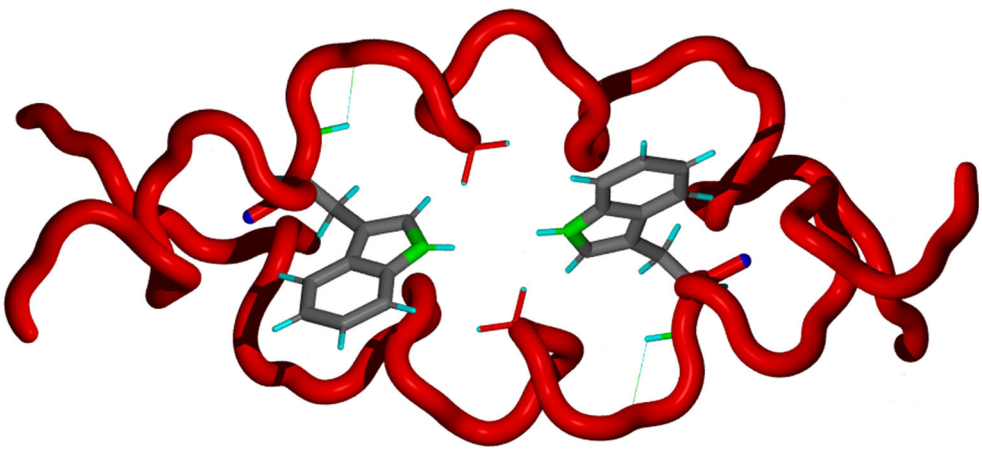

Fig. 8 Dimer of magainin-2 as obtained from an NMR structure investigation in vesicles by Wakamatsu et al. (2002)

"heat up" molecular rotations or re-orientations, leading to proper averaging of the calculated tensor values such that the calculated tensor values agree with the corresponding experimental observations. The tensorial constraints can be further combined with scalar constraints such as distances or isotropic chemical shifts. Concerning the NMR data MDOC provided more conclusive results than full membrane MD simulations, but without the computational burden of having to perform a detailed simulation of the lipids and surrounding water molecules. Due to the fact that MDOC simulations are performed in vacuum they can be completed in relatively short simulation times ($\leq$10 ns), still reaching a complete averaging of the NMR observables. In real time such simulations can be performed in hours or days. Since the method is not limited to rigid molecules and does not depend on the choice of the initial coordinates it is possible to investigate aspects of the structure as dimerization and backbone flexibility. In the MDOC simulation of the PGLa dimer the ends of the helixes unwind and get more mobile.

Simulations on a single PGLa molecule using the orientational constrains from the NMR measurements at the high peptide to lipid ratio of 1:50 revealed that the NMR data allow jumps of the molecule into an orientation nearly parallel to the membrane normal. The simulations with an anti-parallel pair of molecules give rise to a stable dimer that oscillates only weakly around a stable mean orientation. This wobble is comparable to the motions that were observed in single molecule simulation at the lower peptide to lipid ratio of 1:200 (Sternberg et al. 2007) but the overall orientation of the helix axes changed significantly. At lower lipid to peptide ratio the peptide is oriented flat on the membrane surface. At higher concentrations the MDOC simulations provide an orientation which is essential for membrane penetration or pore formation. Since the experimental data are reproduced mostly within the experimental error margins the MDOC simulations confirm the possibility of a membrane spanning state.

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