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Solid state NMR analysis of peptides in membranes: Influence of dynamics and labeling scheme

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ABSTRACT

The functional state of a membrane-active peptide is often defined by its conformation, molecular orientation, and its oligomeric state in the lipid bilayer. These "static" structural properties can be routinely studied by solid state NMR using isotope-labeled peptides. In the highly dynamic environment of a liquid crystalline biomembrane, however, the whole-body fluctuations of a peptide are also of paramount importance, although difficult to address and most often ignored. Yet it turns out that disregarding such motional averaging in calculating the molecular alignment from orientational NMR-constraints may give a misleading, if not false picture of the system. Here, we demonstrate that the reliability of a simplified static or an advanced dynamic data analysis depends critically on the choice of isotope labeling scheme used. Two distinctly different scenarios have to be considered. When the labels are placed on the side chains of a helical peptide (such as a CD_3- or CF_3-group attached to the $C^{\alpha}-C^{\beta}$ bond), their nuclear spin interaction tensors are very sensitive to motional averaging. If this effect is not properly accounted for, the helix tilt angle tends to be severely underestimated. At the same time, the analysis of labels in the side chains allows to extract valuable dynamical information about whole-body fluctuations of the peptide helix in the membrane. On the other hand, the alternative labeling scheme where ^{15}N-labels are accommodated within the peptide backbone, will yield nearly correct helix tilt angles, irrespective as to whether dynamics are taken into account or not.

1. Introduction

Numerous biological processes depend on membrane-bound peptides or transmembrane protein segments, such as antimicrobial defense, membrane fusion, or pore formation [1,2]. As the orientation and assembly of the relevant molecules in the lipid bilayer reflects their functional state, they are often studied by solid state NMR (SSNMR) using suitable isotope labels, yielding a rather static picture of the system [3,4]. Dynamical aspects, however, have not yet received much attention, even though membranes in their physiologically relevant liquid-crystalline phase are highly mobile, and despite the fact that dynamics encodes information about peptide oligomerization and functionality. Motions that are fast on the NMR time scale will lead to a partial averaging of the spin interactions. When such averaging is ignored in calculating the molecular alignment from local orientational constraints, misleading or even completely false results may be obtained. On the other hand, the dynamics found for membrane embedded peptides can not be easily separated from the background dynamics of the lipids. This has been shown to depend, among other things, on sample conditions like hydration [5]. In this respect, using macroscopically oriented bilayers, with low hydration levels, may be advantageous over bicelles or vesicles for the study of peptide dynamics. Nevertheless, in the present study we only consider dynamics of peptides.

Some elaborate models based on relaxation experiments have been used to characterize peptide dynamics in membranes, indicating that they undergo fast axial diffusion (10^{-7}–10^{-8} s) and off-axis reorientations (10^{-5}–10^{-6} s) [6,7]. In addition, it is well known that many membrane-bound peptides undergo rapid rotational averaging around the bilayer normal on the NMR time scale [8–17]. Indeed, when studying peptides at low concentration in liquid crystalline membranes, this motion is generally detected. In macroscopically oriented samples, this averaging has no effect on the NMR parameters when the bilayer normal is oriented parallel to the static magnetic field. However, such motions are manifest when measuring the sample such that the bilayer normal is tilted with respect to the magnetic field. For example, at a 90° sample tilt the fast rotation will average ^{2}H quadrupolar splittings, or ^{1}H–^{15}N and ^{19}F dipolar splittings with a factor of -1/2 compared to the parallel orientation [3,11,14,18,19], and the chemical shift will be affected in a similar

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way [14,18,20]. In systems where this type of motion is present, it is possible to extract the orientational information even from non-oriented samples such as multilamellar vesicles [12,13,21]. However, care must be taken for ^{15}N-NMR studies in unordered samples, since the membrane dynamics and inaccuracies of the ^{15}N CSA tensors may have an important impact on spectra.

Peptides also undergo lateral diffusion in the membrane plane, which has no effect on the NMR parameters of oriented samples. On the other hand, if the tilt angle (τ) or the azimuthal rotation angle (ρ) of the peptide fluctuate, the NMR parameters will be affected [22,23]. Very slow fluctuations, corresponding to a distribution of states on the relevant NMR time scale, will give rise to an overlap of several NMR signals. Fast fluctuations, on the other hand, will lead to an averaging of the observed splittings or chemical shifts. The importance of taking into account such peptide dynamics in the SSNMR structure analysis has recently been noticed in molecular dynamics studies of transmembrane peptides [24,25]. Briefly, if whole-body fluctuations of a peptide are neglected in the analysis, the calculated peptide orientation may be seriously misrepresented [22–25]. To overcome this problem, we have introduced a simple model to account for such whole-body motions. It was thus demonstrated that both, the peptide orientation and the amplitude of helix fluctuations (i.e. fluctuations of τ and ρ angles) in the membrane can be estimated by careful analysis of conventional SSNMR data [22,23].

Here, we describe the differential influence of peptide dynamics on two types of commonly used SSNMR labeling schemes for analyzing peptide orientation, namely on ^{2}H- or ^{19}F-labels in the side chains, and on ^{15}N-labels in the peptide backbone. We perform a systematic study of how whole-body fluctuations will affect the NMR structure analysis in terms of the resulting tilt angle. Using several different dynamical models of varying complexity, this analysis is carried out for the two types of isotope labeling scheme. As it turns out that dynamics has rather different effects in ^{2}H-/^{19}F- and in ^{15}N-NMR, it is possible to take advantage of these differences and choose the adequate labeling scheme, or even to carry out a combined analysis to obtain comprehensive orientational and dynamical information.

2. Methods

2.1. Dynamical models used

Three different models were used to analyze the orientation and dynamics of membrane-bound α-helical peptides. (1) A "static model", where the peptide orientation is described by τ^{fit} and ρ^{fit}, without taking any motions into account; (2) an "implicit dynamical model", where a molecular order parameter S_{mol} is introduced as an additional free parameter, which has the effect of scaling down the calculated splittings due to partial motional averaging, as elaborated previously [13,16,18,19,26–32]; and (3) an "explicit dynamical model", where the mobility of the peptide is described by Gaussian distributions of the τ and ρ angles, with widths of σ_τ and σ_ρ, which describe the extent of whole-body fluctuations of the helix axes about their respective mean angle τ_0 and ρ_0 [22–24].

2.2. Generation of virtual NMR data

As a starting point for the theoretical analysis, a helical peptide of 21 amino acid length was generated using SYBYL (Tripos, St. Louis, MO) with torsion angles of $\varphi = -58°$ and $\psi = -47°$ [18]. The hypothetical isotope labels were placed either onto the $C^{\alpha}-C^{\beta}$ bonds (to generate the virtual ^{2}H-NMR data), or into the amide positions of the backbone (to generate the virtual ^{15}N-NMR data). The virtual NMR parameters were back-calculated for this canonical α-helix considering 8 consecutively labeled positions (nominally from positions 6 to 13 in the center of the helix). The peptide orientation in the membrane was systematically varied in steps of 1° over a range of tilt angles τ^{ref} between 0° and 90°, and keeping the azimuthal angle fixed at $\rho^{ref} = 180°$. The data were produced using the explicit dynamical model 3 defined above, with splittings averaged over Gaussian distributions of τ and ρ, as described previously [22–24].

For the ^{2}H-NMR data, the virtual ^{2}H quadrupolar splittings represent eight 2,2,2-$^{2}H_3$-alanine labels in consecutive positions. A maximum quadrupolar splitting of 84 kHz was used for the rotationally averaged CD_3-group [12,33]. In both the static and the explicit dynamical models, internal peptide motions are taken into account by using a fixed internal order parameter $S^{i} = 0.88$, which effectively reduces the maximum splitting to 74 kHz [23]. For the ^{15}N-NMR data, virtual chemical shifts (CS) and ^{15}N–^{1}H dipolar couplings (DC) were generated for the amide nitrogen at the corresponding labeled positions within the peptide backbone, using tensor values as previously described [22].

2.3. Analysis of the virtual NMR data

Having generated the virtual NMR data based on the explicit dynamics model as outlined above, in the next step we used either the static or the implicit dynamical model to extract the apparent tilt angles τ^{fit} from these NMR data. Since the fits of the ^{2}H- and ^{15}N-NMR data were performed using the same peptide structure, the same definitions of relevant tensors, and the same sets of physical constants as for the generated virtual data, there is no influence in this analysis of any putative peptide conformational flexibility, structural inhomogeneities, or inaccuracy of the theoretical background. Thus the imposed peptide dynamics are of a very specific and well-defined type (Gaussian fluctuations of τ and ρ) and can thus be self-consistently evaluated.

The virtual NMR data was fitted by varying the free parameters (τ^{fit}, ρ^{fit}, and where appropriate S_{mol}) in a grid search and calculating the corresponding NMR observables. The best fits were obtained by minimizing the root-mean-square deviation (rmsd) between the virtual data and the calculated values. For the ^{2}H-NMR data, the rmsd of quadrupolar splittings (in kHz) was used. For the ^{15}N-NMR PISEMA data, the rmsd values for the chemical shifts (CS, in ppm) and for the dipolar couplings (DC, in kHz) were calculated separately, and the total normalized rmsd (rmsd(total)) was minimized, according to:

$$\mathrm{rmsd(total)} = \mathrm{rmsd(CS)/range(CS)} + \mathrm{rmsd(DC)/range(DC)} \quad (1)$$

where the ranges of CS and DC are given by the difference between the maximum and minimum values in the generated set of data. Note that the rmsd of CS and DC have different units, while the total normalized rmsd is unitless.

3. Results and discussion

Virtual NMR data were generated corresponding to peptides that undergo fast whole-body fluctuations. These motions are explicitly described by Gaussian distributions of their tilt (τ) and azimuthal rotation (ρ) angles, centered at τ_0 and ρ_0, and with respective widths of σ_τ and σ_ρ. As representative examples, we consider two distinct situations [23]. One scenario represents a system with "moderate motion", which we describe with appropriate values of $\sigma_\tau = 10°$, $\sigma_\rho = 20°$. This is the case we observed for the amphiphathic peptide PGLa in its inserted (I) oligomeric state [23], and is likely the kind of dynamics to be expected for other oligomeric transmembrane peptides. The second scenario represents a situation of "vigorous motion", as we found for the transmembrane model peptide WALP23, which is characterized here using the values of $\sigma_\tau = 20°$, $\sigma_\rho = 70°$. This second type of dynamics is most likely characteristic of monomeric peptides in a transmembrane orientation. Amphipathic peptides bound at the membrane interface, even in a monomeric

state, can be expected to have moderate to intermediate dynamics, since the rotation around the helix axis and tilt fluctuations are expected to be restricted due to the high free energy of polar and charged side chains when they are exposed to the membrane hydrophobic interior. We have found this latter case for PGLa at low peptide/lipid molar ratios (1/200, S-state) [23].

To generalize the analysis, the virtual NMR data were systematically produced for helices with any tilt angle τ_0^{ref} between 0° and 90°. This helix alignment was then back-calculated as τ^{fit} from the virtual data, by fitting with the two most commonly used models in the literature: (1) a "static model" assuming no whole-body motions of the peptide at all, based on only two parameters τ and ρ; and (2) a "implicit dynamics model" where an additional molecular order parameter S_{mol} is introduced into the fit, which scales down all the underlying NMR interactions by a factor $0 \leq S_{mol} \leq 1$. The advantage of these two simple models is the small number of free parameters in the fit, hence only few experimental data points are needed to determine the helix alignment, e.g., from selective labels.

Fig. 1 shows the result of fitting virtual ^{2}H-NMR data corresponding to a helix labeled with eight discrete 2,2,2-^{2}H-alanine side chains (Ala-d_3). This method, sometimes called geometric analysis of labeled alanines (GALA) has been used in numerous studies of membrane-bound peptides [11–13,16,26,27,34–38]. An equivalent picture as in Fig. 1 is obtained for rigid ^{19}F-NMR labels such as 4-CF_3-phenylglycine, which has been recently introduced as a more sensitive alternative for membrane-bound peptides [13,16–19,28–32,39]. The panels on the left hand side of Fig. 1A–D illustrate the effect of moderate motion upon back-calculating the peptide alignment. Both the static model (dashed line) and the implicit dynamical model (solid line) tend to slightly underestimate the tilt

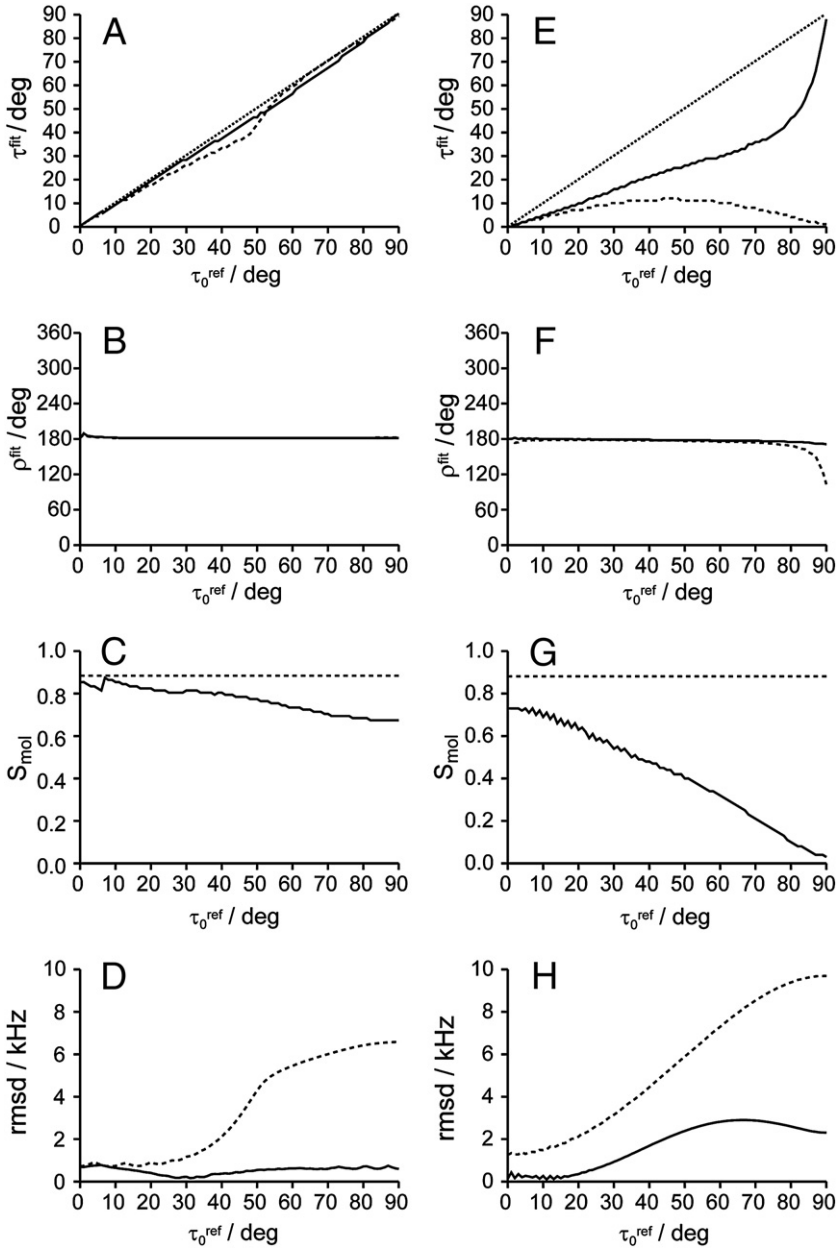

Fig. 1. Fit of virtual ^{2}H-NMR data to the static model (dashed line) or to the implicit dynamical model (solid line), as a function of the real tilt angle τ_0^{ref}. The dotted line shows the ideal case of $\tau^{fit} = \tau_0^{ref}$. (A–D) Moderate motions: $\sigma_\tau = 10°$, $\sigma_\rho = 20°$. (E–H) Vigorous motions: $\sigma_\tau = 20°$, $\sigma_\rho = 70°$. (A, E) Fitted helix tilt angle τ^{fit}. (B, F) Fitted azimuthal angle ρ^{fit}. (C, G) Best-fit S_{mol} values. The dashed line shows the value of $S_{mol} = 0.88$ used in the static model. (D, H) Rmsd values corresponding to the best fits.

angle, but even the static model remains accurate within 10°, as seen in Fig. 1A. Both models reproduce the rotation angle ρ correctly (Fig. 1B). The implicit dynamical model identifies dynamics by a decrease in the S_{mol} value. However, the fitted values of S_{mol} do not reflect the total dynamics correctly, as they vary with τ_0^{ref} (Fig. 1C); even though the mobility used in the generation of the data is the same for all tilt angles, this model interprets the data as if peptides with larger tilt angles were more mobile. Importantly, the static model gives very large rmsd values for $\tau_0^{ref}>40°$ (Fig. 1D). Thus, even if the error in the fitted tilt angle is small, the large rmsd gives a warning that the model is not a good description of the system, i.e. that dynamics is present. For the implicit dynamical model, the rmsd value is small in all cases. It can be noted that there is no correlation between the rmsd values and the errors in τ^{fit}. In some cases, the static model shows a larger rmsd but still gives a τ^{fit} value closer to the reference tilt than the implicit dynamical model.

In the second scenario of vigorous motion (Fig. 1E–H) it is remarkable to see that both models suffer from serious shortcomings. The static model breaks down completely: whatever the real tilt angle τ^{ref} is, the static model will yield a very small value of τ^{fit}, corresponding to an almost upright transmembrane alignment (see Fig. 1E). For instance, a peptide with $\tau_0^{ref}=90°$ will appear to give a best-fit tilt of 0°. Close to $\tau_0^{ref}=90°$ also the ρ^{fit} value deviates significantly from the real value for the static model (Fig. 1F). The slight deviation close to 0° tilt is not important, since ρ is undefined when $\tau=0°$. In many cases the rmsd of the fit remains close to the experimental error, hence the inadequacy of the model cannot be easily noticed (Fig. 1H), except for the static model and for $\tau^{ref}>30°$, where the rmsd soon increases to unrealistic values. In addition, with the implicit dynamical model unrealistically low S_{mol} values are found for large values of τ_0^{ref} (Fig. 1G), a symptom of model failure.

The problems in analyzing NMR data in the presence of whole-body dynamics can be readily rationalized by the geometry of the ^{2}H- or ^{19}F-labels in the peptide side chains (see Fig. 2). For a membrane-embedded helix, the quadrupole splitting of a CD_3-group attached to the C_α position depends on the angle between the C_α-CD_3 bond and the membrane normal. As it happens, the C_α-CD_3 bond forms an angle with the helix axis of ~59° (as does a CF_3-label). Thus, for small tilt angles of typical transmembrane helices, all splittings will be very small, since all labels are oriented close to the magic angle (54.7°) with respect to the membrane normal (Fig. 2A). Likewise, but for a completely different reason, small splittings will also be observed for a highly mobile peptide (Fig. 2C), whatever its average orientation, because the splittings are motionally averaged to small values. As a consequence, when the splittings of a vigorously mobile peptide are fitted with a model that does not take dynamics adequately into account, the best-fit tilt angle will always appear to be very small, as if the helix was aligned transmembrane. Although the implicit dynamical model can account for motional averaging to some extent, helix fluctuations in membranes are inherently anisotropic, which are not properly captured by a scaling factor and can lead to a considerable underestimation of τ^{fit} (Fig. 1E).

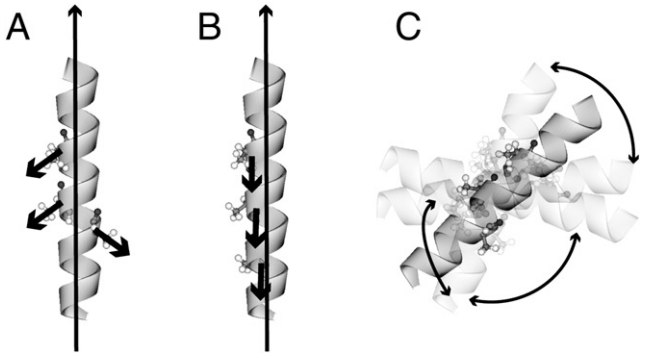

Fig. 2. Orientation of the relevant NMR interaction tensors for ^{2}H- (or ^{19}F-) and ^{15}N-labels. (A) In a rigidly labeled side chain the quadrupolar tensor is directed along the C_α-C_β bond, which is oriented close to the magic angle with respect to the helix axis. (B) In the peptide backbone the ^{1}H–^{15}N dipolar tensor and the ^{15}N CSA tensor are aligned almost parallel to the helix axis. (C) Peptide motion will partially average all tensor values.

In contrast to ^{2}H- or ^{19}F-labels in the peptide side chains, ^{15}N-labels in the backbone have a dipolar and a chemical shift tensor that is oriented virtually parallel to the helix axis (Fig. 2B). Both these NMR interactions can be conveniently analyzed, e.g., in the polarization inversion spin exchange at the magic angle (PISEMA) experiment [40,41]. In these 2D spectra, the peptide orientation in the membrane can be determined directly from the position of the polarity index slant angle (PISA) wheels [42,43]. The effects of dynamics on PISA wheels have been investigated in several studies [22,44–47]. An order parameter will scale both the chemical shift and the dipolar coupling [46,47]. Rotational fluctuations in ρ around the helix axis have been shown to change the size of a PISA wheel, but not the position of its center, hence the correct tilt angle can be recovered even in case of vigorous motion [22,46]. Fluctuations of the tilt angle τ have a more significant impact on the position of the PISA wheel [22,46]. In this case, the smallest splittings for a static orientation are obtained when the helical axis is close to the magic angle, and thus vigorously mobile peptides will give fits that overestimate and underestimate, respectively, small and large mean tilt angles, while tilt angles around 55° are well reproduced [22].

Fig. 3 shows the deviations encountered when fitting virtual PISEMA data to a static model, in full agreement with the previous theoretical considerations. For moderately mobile peptides (Fig. 3A–D) the static model is perfectly sufficient. For vigorously mobile helices (Fig. 3E–H) there are noticeable deviations for τ^{ref} close to 0° and 90°, while good fits are obtained from 30° to 60° (Fig. 3E). The rmsd, especially for CSA, is larger in the case of vigorous motions, which is a symptom of dynamic averaging, while the dipolar rmsd remains at the level of the experimental error (see Fig. 3G and H). However, there is no correlation between rmsd values and deviations in calculated and real tilt angles, hence rmsd is not a quantitative indicator of the quality of the fit.

4. Summary and conclusions

Considering the impact of dynamics on the analysis of commonly used SSNMR data, and noting the distinct influence of the geometry of the reporter group, it is possible now to select the most appropriate labeling scheme and dynamical model to investigate a peptide in any particular situation. ^{2}H-NMR analysis requires specific labeling, but has the advantage of allowing very simple experiments. ^{19}F is convenient due to its high sensitivity, which makes it useful at low peptide concentration, where other nuclei fail to give detectable signals. For example, amphipathic peptides tend to lie on the membrane surface with moderate mobility, hence their orientation can be accurately determined by ^{19}F-NMR using the implicit dynamical model [23]. At high concentration, peptide oligomers may form, which are expected to reduce whole-body dynamics, so also in this case the implicit dynamical model will give accurate orientational values. Note, however, that when oligomerization is expected it is best to use non-disturbing labels, such as ^{2}H (for native Ala positions) or ^{15}N.

According to our assessment above, ^{15}N-labeling is recommended in cases where vigorous dynamics are expected. Peptides can be readily and uniformly labeled with ^{15}N by biosynthesis, though specific ^{15}N-labeling is usually required in order to determine the azimuthal rotation angle. The technically demanding 2D PISEMA experiment is often combined with this labeling scheme. For monomeric transmembrane peptides, which have repeatedly been found to be vigorously mobile, this appears to be the method of choice

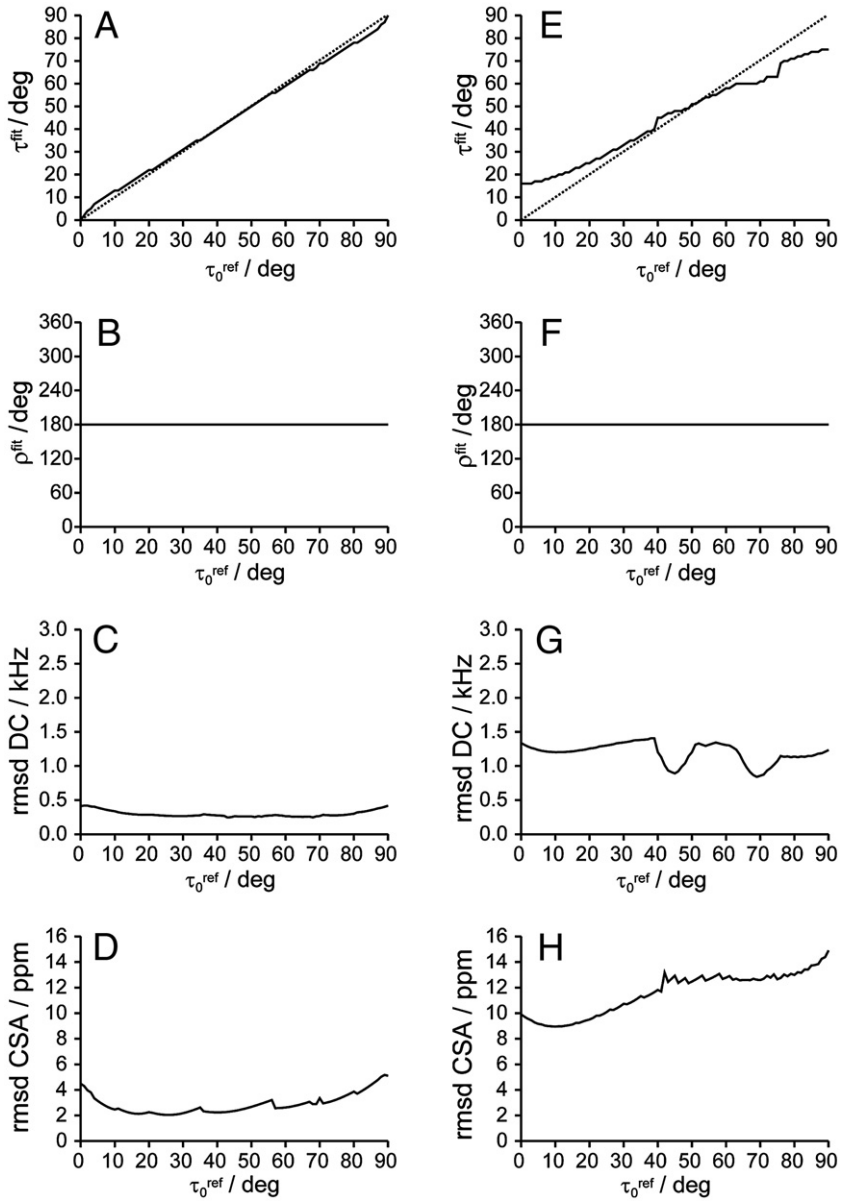

Fig. 3. Fit of virtual ^{15}N-NMR PISEMA data using the static model (solid line), as a function of the real tilt angle τ_0^{ref}. The dotted line shows $\tau^{fit} = \tau_0^{ref}$: (A–D) Moderate motions: $\sigma_\tau = 10°$, $\sigma_\rho = 20°$. (E–H) Vigorous motions: $\sigma_\tau = 20°$, $\sigma_\rho = 70°$. (A, E) Fitted helix tilt angle τ^{fit}. (B, F) Fitted azimuthal angle ρ^{fit}. (C, G) Rmsd values for the ^{1}H-^{15}N dipolar coupling of the best fits. (D, H) Rmsd values for the ^{15}N chemical shift of the best fits.

for finding the helix orientation. In contrast, labeling with ^{2}H or ^{19}F in the side chains appears to be risky and is likely to give significantly underestimated helix tilt angles in transmembrane peptides, as the effect of motional averaging clearly requires the use of explicit or even more advanced dynamical models [23]. The risk, as we have explained, is less for moderately mobile oligomers and surface-bound monomers. Additionally, whenever possible, enough selective isotope labels should be used to allow for an analysis with the explicit dynamic model. On the other hand, static models can be quite safely used to analyze ^{15}N-PISEMA data, as vigorous fluctuations in ρ do not affect the fitted tilt angle, and fluctuations in τ may impose moderate deviations only. For some cases of oligomeric peptides, it was found that including dynamics in the analysis did not improve the fit of PISA wheels, and in these systems with low peptide mobility a static model is appropriate [46,47]. Yet, for highly mobile ^{15}N-labeled peptides it is nevertheless recommended to use explicit dynamics models, as these can provide additional information about the amplitudes and anisotropy of the underlying whole-body motions [22]. The same perspective holds for ^{2}H- and ^{19}F-labels in the side chains, as these labeling schemes are optimally suited to extract dynamical information of membrane-bound helical peptides.

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