

REDOR NMR on a Hydrophobic Peptide in Oriented Membranes

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Received May 22, 2000; revised July 31, 2000

A method is presented for the calculation of REDOR dephasing for specifically labeled membrane-spanning peptides in uniformly aligned lipid bilayers under magic angle oriented sample spinning (MAOSS) conditions. Numerical simulations are performed for dephasing of ¹³C signal by ¹⁵N when the labels are placed in an α -helical peptide at the carbonyl of residue (*i*) and amide nitrogen of residue (*i* + 2) to show the dependency of REDOR echo intensity on the peptide tilt angle relative to the membrane normal. The approach was applied to the labeled transmembrane domain of phospholamban (¹⁵N-Leu₃₇, ¹³C-Leu₃₉]PLB_{TM}) incorporated into dimyristoylphosphatidylcholine bilayers. The dephasing observed for a random membrane dispersion showed that the peptide was α -helical in the region including the two labels, and dephasing in oriented membranes showed that the peptide helix was tilted by $25^\circ \pm 7^\circ$ relative to the bilayer normal. These results agree with those obtained by other spectroscopic methods.

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Key Words: MAOSS; phospholamban; magic-angle spinning; membrane; peptide.

Several solid-state NMR methods have emerged over the past decade for the structural analysis of partially or uniformly isotopically labeled biomolecules in lipid membranes (1). These methods are, in general, based either on magic-angle spinning (MAS) approaches in which second-rank interactions are removed by sample rotation and then partially reintroduced by radiofrequency pulses or adjustment of the sample spinning frequency (2), or on methods that exploit tensor orientations in uniaxially aligned lipid bilayers (3). A recent advance has been to combine the two approaches as magic-angle *oriented* sample spinning (MAOSS), in which MAS experiments are performed on membranes aligned on glass plates to provide a highly sensitive method for determining molecular orientations of membrane samples, such as the retinal chromophore in bacteriorhodopsin (4, 5).

A major goal in solid-state NMR is to solve the structures of uniformly (¹³C, ¹⁵N) isotope labeled proteins and peptides in membrane environments. Significant progress is being made toward this aim, as issues of spectral resolution, assignment,

and quantitative analysis of multiple-spin systems are being addressed (6). Nevertheless, solid-state NMR methods that place small numbers of localized structural constraints on specifically labeled membrane peptides and proteins continue to be valuable and informative alternatives to complete structure determination (7). These methods involve either specifically measuring internuclear distances using dipolar recoupling methods (e.g., REDOR or rotational resonance), or bond angles relative to a unique director axis, usually the membrane normal. Such methods provide a limited amount of model-independent structural information with high precision.

This work seeks to increase the amount of structural information that can be obtained from selectively labeled biomolecules by adapting the MAOSS method to provide both distance and angular information from specifically isotope labeled peptides in lipid bilayers. In so doing the aim is to determine from the same labeling scheme (i) the local secondary structure of the peptide in the region between the two labels and (ii) the tilt angle of the peptide long axis in the lipid bilayer. If successful, the approach could be extended to examine the structure and relative orientations of membrane-spanning regions in larger polytopic membrane proteins.

The experimental strategy is based on selectively measuring dipolar couplings from a labeled peptide in a randomly dispersed membrane and in a lipid bilayer uniformly oriented on glass plates, in both cases while applying magic-angle spinning. In the membrane dispersion the measured dipolar coupling constant ν_D , which is inversely proportional to the cube of the internuclear distance r_{IS} between the labeled sites, is diagnostic of the local secondary structure. In an oriented sample, the distance-dependent value of ν_D is also sensitive to the angle between the internuclear vector and the bilayer normal, and hence to the tilt axis of the peptide in the membrane (Fig. 1). The heteronuclear recoupling method REDOR was used to measure ν_D because it does not require fast spinning, which could potentially disrupt sample orientation, and because the experimentally observed signal dephasing is related only to the magnitude and orientation of the dipolar vector (8). Hence, the analysis of dephasing curves is simple and does not rely on knowledge of the relative orientations of chemical shift tensors or on multiple-quantum relaxation times, which can be difficult to determine accurately (9).

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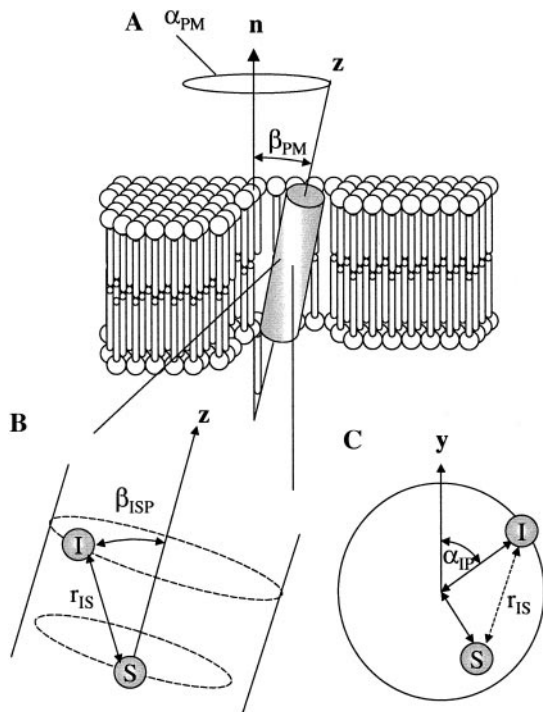


FIG. 1. Definition of geometric terms for an I-S spin pair in a membrane-spanning peptide distributed within in a perfectly uniaxially oriented lipid bilayer (no mosaic spread). (A) The tilt of the peptide relative to the membrane normal \mathbf{n} is described by polar angle β_{PM} , and the peptide distribution traces a cone about \mathbf{n} described by azimuthal angle α_{PM} . (B) The orientation of an I-S internuclear vector of length r_{IS} is defined in an axis system with z parallel to the peptide long axis, with β_{ISP} defining the angle between the I-S vector and z . (C) Viewed along the peptide long axis, angle α_{IP} defines the rotational orientation of the peptide about its long axis in terms of the position of spin I.

In the REDOR method the signal from spin I (e.g., ^{13}C) is measured in two experiments, one with dephasing π pulses placed at the center of each rotor cycle at the frequency of spin S (e.g., ^{15}N), the other without dephasing pulses. Dipolar dephasing after N_c rotor cycles at a spinning frequency ν_R is quantified as S/S_0 , which is the ratio of the echo intensity after dephasing π pulses (S) to the full echo intensity without π pulses (S_0). In the case of a powder sample or membrane dispersion, the observed REDOR dephasing is calculated from (10)

$$\frac{S}{S_0} = \frac{1}{4\pi} \int_{\alpha=0}^{2\pi} \int_{\beta=0}^{\pi} \cos \left[\frac{\nu_D}{\nu_R} 2\sqrt{2} \sin 2\beta \sin \alpha \right] \sin \beta d\alpha d\beta. \quad [1]$$

The polar and azimuthal angles, α and β , respectively, define the orientation of individual heteronuclear dipolar vectors in the fixed MAS rotor axis system, and standard powder averaging is performed over all values of α and β .

The case is now considered of a perfectly oriented lipid

bilayer with the director axis \mathbf{n} (the bilayer normal) aligned along the magic angle and containing a labeled membrane-spanning peptide orientated at unique angle β_{PM} relative to the bilayer normal (Fig. 1). The internuclear distance vector r_{IS} separating a single heteronuclear I-S spin-pair in the peptide is not isotropically distributed as in a powder sample (i.e., Eq. [1]), but takes restricted orientations dependent on β_{PM} and azimuthal angle α_P , which defines the rotational orientation of the peptide about its long axis. The angle β_{ISM} between the I-S internuclear vector and the bilayer normal is calculated from

$$\beta_{ISM} = \cos^{-1}[\cos \beta_{ISP} \cos \beta_{PM} - \cos \alpha_{IP} \sin \beta_{ISP} \sin \beta_{PM}], \quad [2]$$

where β_{ISP} is the angle between z and the I-S vector (Fig. 1) and angle α_{IP} defines the rotational excursion of I from a reference axis y perpendicular to the rotation axis (Fig. 1). REDOR dephasing can now be calculated for any peptide tilt angle in a uniaxially oriented membrane sample, according to

$$\begin{aligned} \frac{S}{S_0}(\alpha_{IP}, \beta_{PM}) &= \frac{1}{4\pi} \int_0^{2\pi} \cos \left[\frac{\nu_D}{\nu_R} 2\sqrt{2} \sin 2\beta_{ISM} \sin \alpha_{PM} \right] d\alpha_{PM}, \quad [3] \end{aligned}$$

where α_{PM} is the azimuthal angle defining the peptide distribution about the membrane normal (Fig. 1).

In practice, an aligned membrane sample has a distribution of molecular orientations about the average membrane normal \mathbf{n} . The resulting distribution of peptide molecules, with tilt angles β_{PM} oriented about a mean tilt angle $\bar{\beta}_{PM}$, is taken into account by modification of Eq. [3] with a Gaussian distribution function (11)

$$F(\beta_M) = N \int_0^{\pi} \exp \left[-\frac{(\bar{\beta}_{PM} - \beta_{PM})^2}{2\Delta\beta^2} \right] \sin \beta_{PM} d\beta_{PM}, \quad [4]$$

where N is a normalization constant and $\Delta\beta$ is the macroscopic mosaic spread of the bilayer.

This strategy was used to examine the 24-residue transmembrane domain of the cardiac contractility regulating protein phospholamban labeled with ^{13}C at the carbonyl of Leu₃₇ and ^{15}N at Leu₃₉ (^{15}N -Leu₃₇, ^{13}C -Leu₃₉)PLB_{TM} and incorporated into dimyristoylphosphatidylcholine (DMPC) membranes. The three cysteines of wild-type phospholamban were substituted with alanine to prevent self-association of the peptide in the membrane. The ^{13}C - ^{15}N internuclear distance in this (i), ($i + 2$) labeling scheme is diagnostic of secondary structure and is predicted to be 3.26 Å for an α -helix and 4.16 Å for a β -sheet.

The analysis of REDOR and other dipolar recoupling experi-

ments on randomly dispersed and oriented membrane peptides must take into consideration whether the peptide rotates about its long axis in the membrane. Fast molecular rotational diffusion (on the homonuclear and heteronuclear dipolar coupling timescales) will reduce the rates of Hartmann–Hahn cross-polarization and, in dipolar recoupling experiments, scale the observed signal dephasing. In the case of peptides that self-associate into channels, and peptides and proteins containing hydrophilic domains outside the lipid bilayer, the axial rotation of individual membrane domains may be hindered by protein–protein contacts (12) or protein–lipid interactions (8). On the other hand, shorter monomeric peptides like PLBTM are more likely to rotate freely about their long molecular axis when in fluid lipid membranes because fewer opportunities exist for motional restraint of the peptide. Moreover, if the peptide is tilted with respect to the membrane normal, the peptide long axis may also rotate about the membrane normal (13).

It is important to assess the rotational mobility of individual peptides before REDOR dephasing can be analyzed. Lineshape analysis of static broad line ^2H NMR spectra of $[\text{CD}_3\text{-Ala}]$ PLBTM in fluid phase DMPC bilayers (above 23°C) showed that the averaging of the ^2H quadrupolar interaction was indeed consistent with axial rotation of PLBTM with a correlation time of 10^{-4} – 10^{-5} s, whereas no evidence for molecular rotational averaging was observed below -20°C (unpublished results). This does not appear to be the case, however, in ^2H spectra of the EGF receptor transmembrane domain in DOPC bilayers (13). In this case the residual quadrupole splittings suggested that the peptide rotates about the membrane normal, but does not rotate about its long axis and is described by a unique orientational angle.

In the REDOR experiments described in this work, it was desirable to fully eliminate the scaling effects induced by axial rotation of PLBTM, and this was achieved by freezing both oriented and unoriented samples at -50°C . It is assumed that freezing out the free axial rotation of PLBTM produces an ensemble of peptide rotamers, which are defined by rotational angles α_{IP} (Fig. 1) distributed randomly between 0° and 360° . Such a rotamer distribution does not affect REDOR dephasing in a randomly dispersed membrane, but for an aligned membrane sample Eq. [3] must be modified to perform averaging over the full distribution of rotational angles α_{IP} , to give

$$\begin{aligned} \frac{S}{S_0}(\beta_{\text{PM}}) &= \frac{1}{4\pi^2} \int_{\alpha_{\text{PM}}=0}^{2\pi} \int_{\alpha_{\text{IP}}=0}^{2\pi} \left[\frac{\nu_{\text{D}}}{\nu_{\text{R}}} 2\sqrt{2} \sin 2\beta_{\text{ISM}} \sin \alpha_{\text{PM}} \right] d\alpha_{\text{PM}} d\alpha_{\text{IP}}. \end{aligned} \quad [5]$$

If the peptide is tilted in the membrane, freezing the sample may give an additional ensemble of peptides distributed about

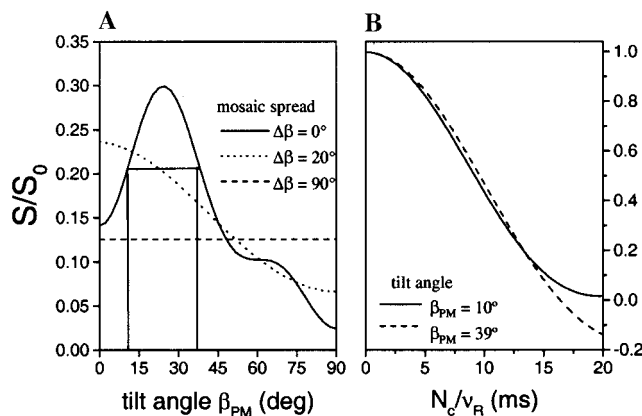


FIG. 2. Calculated ^{15}N -dephasing of the ^{13}C signal from an α -helical peptide tilted at an angle β_{PM} in a uniaxially oriented lipid bilayer, with labels placed 3.28 \AA apart in the carbonyl of residue (i) and amide of residue ($i + 2$). (A) The extent of dephasing at peptide tilt angles β_{PM} ranging from 0° to 90° was calculated for mosaic spreads $\Delta\beta$ of 0° (solid line), 20° (dotted line), and 90° (dashed line). All curves represent the fraction of signal (S/S_0) remaining after 40 rotor periods (N_c) at a MAS frequency ν_R of 3000 Hz. The interpolating lines show examples of two tilt angles ($\beta_{\text{PM}} = 10^\circ$ and 39°) giving identical values of S/S_0 under these conditions when $\Delta\beta = 0^\circ$. (B) Calculated dephasing curves ($\Delta\beta = 0^\circ$) covering a range of echo times (N_c/ν_R) from 0 to 20 ms for peptide tilt angles of 10° (full lines) and 39° (dashed lines).

the membrane normal (Fig. 1). This possibility is already covered in Eq. [3] and Eq. [5] by performing averaging over α_{PM} .

The dependence of ^{15}N -dephased ^{13}C echo intensity on the peptide tilt angle (β_{PM}) was calculated from Eq. [5] for a perfectly aligned ($\Delta\beta = 0^\circ$) α -helical membrane peptide labeled as for ^{15}N -Leu $_{37}$, ^{13}C -Leu $_{39}$]PLBTM (Fig. 2A; solid line). Signal dephasing is clearly sensitive to β_{PM} , with S_0/S ranging from 0.3 to 0.03 as the tilt angle β_{PM} increases from 0° (helix axis parallel to the membrane normal \mathbf{n}) to 90° (helix axis perpendicular to \mathbf{n}). The solid curve in Fig. 2A shows that unique values of S/S_0 are not observed when the tilt angle falls between 0° and 45° , which unfortunately is the range of angles seen for most membrane peptides. Within this range of β_{PM} , any single value of S_0/S at the given echo time ($N_c/\nu_r = 13$ ms) is consistent with two possible peptide tilt angles. For example, a measured value of S/S_0 of 0.20 would be consistent with peptide tilt angles of 10° and 39° when the mosaic spread is close to zero (Fig. 2A).

This ambiguity in the tilt angles determined from S/S_0 (Fig. 2A; solid line) can be avoided by comparing full REDOR dephasing curves over a series of echo times. The calculated curves in Fig. 2B show that while there is little to distinguish the signal dephasing for $\beta_{\text{PM}} = 10^\circ$ (solid line) and $\beta_{\text{PM}} = 39^\circ$ (dashed line) when the echo time is short (< 15 ms), the curves diverge significantly at longer times. Hence, with appropriate selection of echo times it is possible to identify a unique peptide tilt angle from the signal dephasing. Introducing a mosaic spread of 20° slightly reduces the sensitivity of dephasing to the full range of possible peptide tilt angles (Fig. 2A;

dotted line) but, crucially, each value of S_0/S within the upper and lower limit now corresponds to a unique peptide tilt angle. With a mosaic spread of 90° , the echo intensity is no longer dependent on β_{PM} (Fig. 2A; dashed line) and REDOR dephasing is equivalent to that expected for a random membrane dispersion.

A series of ^{13}C observe, ^{15}N -dephased REDOR experiments (operating field strength of 11.7 T) was carried out at -50°C on disperse DMPC membrane samples containing [$^{15}\text{N}\text{-Leu}_{37}$, $^{13}\text{C}\text{-Leu}_{39}$]PLBTM and on the same membrane samples oriented on a stacked set of 5 mm diameter glass disks. The static ^{31}P spectrum of the oriented membranes (with the glass plates perpendicular to the magic-angle) indicated that the membranes were well aligned (Fig. 3A; right), and a lower limit on the mosaic spread ($\Delta\beta = 10^\circ$) was estimated directly from the ^{31}P linewidth. It was not possible to determine $\Delta\beta$ more precisely by an independent method.

The carbonyl region of typical ^{13}C REDOR echo spectra for [$^{15}\text{N}\text{-Leu}_{37}$, $^{13}\text{C}\text{-Leu}_{39}$]PLBTM in oriented membranes is shown in Fig. 3B and values of S/S_0 measured from the peptide carbonyl peak for disperse and oriented membrane samples are shown in Fig. 3C (left). In the case of the membrane dispersion, curves were calculated from Eq. [1] and the best fit to the experimental data was obtained for a dipolar coupling constant ν_D of 98 Hz. The value of ν_D corresponds to a through-space $^{13}\text{C}_{(i)}\text{-}^{15}\text{N}_{(i+2)}$ distance of $3.2 \pm 0.2 \text{ \AA}$, which is close to the distance of 3.26 \AA expected for an α -helical structure. This conclusion agrees with the results from rotational resonance NMR (14) and FTIR (15) measurements on the same peptide sequence, which also indicate an α -helix.

Simulations of the REDOR dephasing observed for [$^{15}\text{N}\text{-Leu}_{37}$, $^{13}\text{C}\text{-Leu}_{39}$]PLBTM in the oriented membrane sample were performed by substituting the predetermined value of ν_D into Eq. [5] and varying β_{PM} and $\Delta\beta$ to find the distribution of peptide tilt angles giving curves best fitting the experimental data. Curves were calculated for peptide tilt angles of between 0° and 50° and, because the mosaic spread could not be measured accurately, the calculations were also performed for a range of $\Delta\beta$ values above 10° (the lower limit estimated from ^{31}P spectra). χ^2 analysis, represented by the two-dimensional plot in Fig. 3C (right), showed that the experimental data could not be simulated from unique values of β_{PM} and $\Delta\beta$, but instead the best fitting curves were found when the mosaic spread was between 10° and 19° and the peptide tilt angle was $25^\circ \pm 7^\circ$. The range of tilt angles found here compares favorably with the angle of around 30° found independently by other spectroscopic methods (16).

The simple case of a short, single membrane spanning peptide containing an isolated spin-pair has been used here to demonstrate how MAOSS can be applied to obtain information about both the secondary structure and orientation of selectively labeled peptides and proteins in biomembranes. Although uncertainties in the molecular distribution about the director axis (i.e., the mosaic spread $\Delta\beta$) reduce the precision

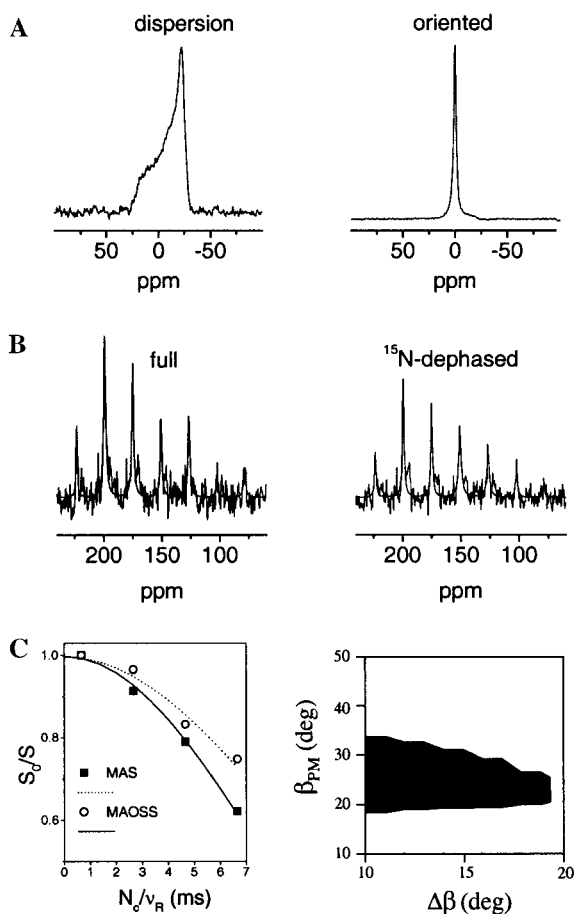


FIG. 3. NMR spectra, signal intensities, and simulations of REDOR dephasing for [$^{15}\text{N}\text{-Leu}_{37}$, $^{13}\text{C}\text{-Leu}_{39}$]PLBTM in DMPC membranes (at a lipid-to-peptide mole ratio of 20:1), prepared as a random membrane dispersion or oriented on glass disks by slow rehydration. (A) Static, proton decoupled ^{31}P NMR spectra from a random dispersion (left) and a sample of stacked disks aligned at the magic angle (right). (B) Carbonyl peak intensities in full-echo (left) and ^{15}N -dephased echo (right) ^{13}C spectra of oriented membranes at -50°C , obtained after 20 rotor cycles (N_c) at a MAS frequency of 3000 Hz. (C) Experimental and simulated ^{15}N dephasing of the carbonyl ^{13}C signal (S/S_0) from peptide in the membrane dispersion (■) and the oriented sample (□) observed at 4 echo times (right). Data from the random dispersion was simulated using Eq. [1] (solid line) from which a distance of 3.2 \AA was determined. Data from the oriented sample was simulated for an α -helical geometry using Eq. [5] (dotted line). In the simulation procedure, curves were calculated by varying the values of β_{PM} and $\Delta\beta$ to find the best fit to the experimental data (χ^2 minimum). The best fits are shown as a dark region in the two-dimensional plot (right) and are consistent with a peptide tilt angle β_{PM} of $25^\circ \pm 7^\circ$.

with which β_{PM} can be measured (a limitation of all experiments on oriented membrane systems), the REDOR method is simple and the information can be interpreted without knowledge of tensor orientations or relaxation rates (9). It is envisaged that by incorporating several spin-pairs in remote regions of larger membrane proteins, the combined REDOR-MAOSS approach could be useful in determining the relative orientations of two or more membrane spanning helices.

ACKNOWLEDGMENTS

The authors thank Professor Anthony Lee and Dr. Ram Sharma for synthesis and purification of phospholamban peptides. BBSRC is acknowledged for a Senior Research Fellowship (to A.W.), a CASE studentship with SmithKline Beecham Pharmaceuticals (to Z.A.), and equipment awards (with HEFCE) under the JREL.

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