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# Simulation of NMR Data from Oriented Membrane Proteins: Practical Information for Experimental Design

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ABSTRACT Several hundred solid state NMR dipolar couplings and chemical shift anisotropies were simulated for the polytopic membrane protein, bacteriorhodopsin, and for an idealized transmembrane peptide conforming to several different secondary structures ($\alpha$- and $3_{10}$-helices and parallel and antiparallel $\beta$-sheets), each at several tilt angles with respect to the bilayer normal. The use of macroscopically oriented samples was assumed. The results of these simulations suggest:

(i) Because of the $r^{-3}$ dependence of dipolar coupling, it is likely to prove difficult to successfully execute *uniform* isotopic enrichment strategies to generate large numbers of quantitatively interpretable structural measurements in oriented sample NMR studies of membrane proteins.

(ii) There are a number of readily implementable *specific* isotopic labeling schemes which can yield data patterns sufficient to identify local secondary structure for transmembrane segments of idealized proteins which are tilted by $<10°$ with respect to the bilayer normal.

(iii) The measurement of dipolar coupling constants between $^{13}C$-, $^{19}F$-, and/or $^{3}H$-labeled side chains of proximal residues may prove effective as routes to long range tertiary structural data constraints.

## INTRODUCTION

The determination of the three-dimensional structure of a membrane protein by *any* method generally remains a formidable problem. In principle, solid state nuclear magnetic resonance (SSNMR) should be capable of such a task (Opella and Stewart, 1989; Brenneman and Cross, 1990; Smith and Peersen, 1992). However, this area has traditionally been limited by the poor spectral resolution of powder patterns arising from disoriented membrane samples.[1] This problem has been dealt with by the development of rapid sample spinning and sample orientation methods which eliminate powder patterns and yield high resolution spectra from anisotropic membrane phases (e.g., Smith and Peersen, 1992; Moll and Cross, 1990; Cornell et. al., 1988; Sanders and Prestegard, 1990). As a result of the increased accessibility of structurally useful data afforded by such "high resolution" SSNMR methods, the determination of three-dimensional structures of moderately sized membrane proteins from SSNMR data is now feasible.

The success of solution NMR as a route to protein structure determination is based upon the ability of multidimensional NMR to provide large numbers of structural constraints for the protein of interest (Wuthrich, 1986). Like solution NMR, "magic angle" sample spinning NMR techniques such as REDOR (Gullion and Schaefer, 1989) and rotational resonance (Levitt et al., 1990) can also provide quantitative internuclear distance measurements. However, such techniques have yet to be developed to the point where more than a few measurements can be made in a single, technically demanding set of experiments.[2] At the present, oriented sample NMR (OSNMR) techniques may offer a more facile route to higher numbers of structurally relevant measurements. For example, in studies of lipids it has been demonstrated that by using magnetically oriented model membrane systems, it is possible to readily acquire 10–20 structural constraints using NMR instrumentation and methods which are quite crude by rapid sample spinning technology standards (Sanders, 1993; Sanders and Prestegard, 1991). However, the structural information encoded in OSNMR measurements is not as directly interpretable as in the case of REDOR/rotational resonance data. Nevertheless, the direct use of OSNMR data parameters such as chemical shift anisotropy (CSA), dipolar and quadrupolar couplings in structural studies is now possible through the use of "angle geometry" (Brenneman and Cross, 1990), data-constrained energy minimizations (Ram et al., 1989), and other techniques (Chiu et al., 1991; Hare et al., 1993; Sanders and Schwonek, 1993) which allow tensor orientation-dependent measurements to be translated into conformational, orientational, and dynamic structural information.

The use of OSNMR has now led to the complete elucidation of the backbone structure of membrane-associated gramicidin A, a small ion channel (Mai et al., 1993; Teng et al., 1991; Ketchum et al., 1993). Despite advances made in the course of this impressive work and other stud-

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[1] Of course, powder patterns have their own unique utility in structural studies including a distinctive ability to provide information on molecular dynamics (e.g., Dufourq et al., 1992).



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[2] There is reason to hope this limitation is being overcome. See Bennett et al., 1992.

**TABLE 1 Summaries of predicted numbers and ranges of dipolar couplings for uniformly labeled proteins**

| Identity of target nucleus and the uniform labeling | Total number of coupling predicted | Breakdown of coupling constant ranges (Hz) |
|---|---|---|
| $^1H_\alpha$ of Val-14 of idealized $\alpha$-helix (0° tilt): $^1H$-$^1H$ coupling to all other protons on the 26-residue helix. | 190 | 0–10: 3%<br>10–50: 16%<br>51–500: 65%<br>>500: 17% |
| Thr-89-$^1H_\alpha$ of helix 2 of BR: $^1H$-$^1H$ coupling to all other protons on a juxtaposed helix (helix 3 of BR) | 151 | 0–20: 32%<br>20–100: 30%<br>>100: 38% |
| $^{13}C{=}O_{amide}$ of Thr-89 of BR: $^{13}C$-$^{13}C$ couplings to all other carbons on adjacent (±4) residues of the same helix. | 58 | 2–10: 35%<br>11–70: 45%<br>>70: 19% |
| Met-20 $^{13}C{=}O_{amide}$ and $^{13}C_\alpha$ of helix 1 of BR: couplings to all $^{13}C{=}O_{amide}$ and $^{13}C_\alpha$ of the nearest 12 residues on the adjacent helix (helix 2). | 48 | <5: 27%<br>5–15: 50%<br>>15: 23% |
| $^{13}C{=}O_{amide}$ of Val-14 of idealized $\alpha$-helix (0° tilt): $^{13}C$-$^{13}C$ coupling to all other backbone $^{13}C{=}O$ on same helix. | 25 | 1–10: 48%<br>10–30: 24%<br>>30: 29% |
| $^{13}C{=}O_{amide}$ of Val-14 of idealized parallel $\beta$-sheet (0° tilt): $^{13}C$-$^{13}C$ coupling to all other backbone $^{13}C{=}O$ on same strand. | 25 | <1: 44%<br>1–10: 32%<br>>10: 24% |
| $^{15}N$ of Val-14 of idealized $\alpha$-helix: coupling to all other backbone $^{15}N$ on helix. | 25 | <1: 36%<br>1–5: 36%<br>>5: 28% |

The results of this table can be roughly applied to other possible isotopic combinations by 1) estimating the (total H):(total C), (backbone C):(backbone N), and (total C):(backbone N) ratios in proteins to be roughly 2:1, 2:1, and 7:1, respectively, and 2) bearing in mind that for identical internuclear distances and orientations relative $^1H$-$^1H$, $^{13}C$-$^1H$, $^{15}N$-$^1H$, $^{15}N$-$^{13}C$ $^{13}C$-$^{13}C$, and $^{15}N$-$^{15}N$ dipolar couplings will be 100, 25, −10, −2.5, 6.3, and 1.0, respectively.

ies, many possible experimental strategies for studying membrane protein structure using oriented sample NMR remain unexplored. In this paper, some of these strategies are preassessed by *simulating* OSNMR data for both real and idealized transmembrane (TM) proteins. These simulations were designed to provide some insight into the following issues: (i) Can uniform labeling strategies be employed in oriented sample SSNMR to provide large data sets analogous to those generated in solution NMR studies of protein structure? (ii) What *specific* labeling schemes are likely to prove most effective in providing structural data? (iii) In cases where TM segments of proteins are likely to exist as stretches of regular secondary structure, can *data pattern recognition* be used to identify the local structure? (iv) Assuming the conformations and membrane orientations of *individual* TM segments can be determined from SSNMR data, can the intersegmental contacts present in polytopic TM proteins be ascertained from OSNMR data?

While the above issues can only be *definitively* addressed by actual experiments, the results of these simulations may prove useful in rational experimental design, a utility which is perhaps best appreciated when one considers the cost of producing an isotopically labeled polypeptide.

## METHODS

### Proteins for which simulations were made

Data were simulated for bacteriorhodopsin (BR) based on its experimentally derived moderate resolution structure (Henderson et al., 1990) and for a set of idealized transmembrane (TM) helices and sheets. Bacteriorhodopsin contains seven transmembrane $\alpha$-helices.

The same sequence for an "idealized" TM segment protein was used for all four secondary structures studied. The arbitrarily chosen sequence was the carboxyl terminus of *Escherichia coli* diacylglycerol kinase (which is probably exists as a TM $\alpha$-helix in its native state, see Loomis et al. (1985)). The sequence of this peptide is as follows.

$H_2N$-Met-Gly-Ser-Ala-$Ala_5$-Val-Leu-Ile-Ala-

$Ile_{10}$-Ile-Val-Ala-Val-$Ile_{15}$-Thr-Trp-Cys-Ile-$Leu_{20}$-

Leu-Trp-Ser-His-$Phe_{25}$-Gly-COOH

The peptide was forced into four different secondary structures ($\alpha$-helix, $3_{10}$-helix, antiparallel $\beta$-sheet, and parallel $\beta$-sheet) using an interactive modeling program, BIOGRAF (Molecular Simulations, Waltham, MA). The four structures were minimized using molecular mechanics (Dreiding II force field (Mayo et al., 1990)) in order to eliminate any energetically unrealistic interactions present in the unrelaxed idealized structures. Each of the four secondary structures was set at five different orientations with respect to the bilayer normal (defined as the $z$ axis): 0°, 2°, 5°, 15°, and 30°. In the case of the $\beta$-sheet structures, the tilts were made (arbitrarily) about a bisector lying in the plane of the sheet "ribbon" and

**TABLE 2 Predicted of CSA (PPM) and dipolar couplings (Hz) for backbone nuclei of residues 12–14 of the idealized transmembrane peptides (untilted)**

| Nucleus on residues 12–14and 2° structure | $^1$H, directly bonded (dipolar)* | CSA (PPM) | $^{15}$N+0 dipolar‡ | $^{15}$N+1 dipolar | $^{15}$N+2 dipolar | $^{15}$N+3 dipolar | $^{15}$N+4 dipolar | $^{13}C_\alpha$+0 dipolar |
|---|---|---|---|---|---|---|---|---|
| $N_{amide}$ | | | | | | | | |
| α-Helix | 22672 to 25926 | 86 to 105 | NA¶ | −6 to 11 | −11 to −5 | −20 to −14 | −11 to −3 | 91 to 474 |
| | L+§ | L+ | | s | s | s | s | L+ |
| $3_{10}$-Helix | 20778 to 20912 | 87 to 97 | NA | −23 to −34 | −23 to −18 | −11 to −11 | −4 to −4 | 483 to 692 |
| | L+ | L+ | | s | s | s | s | L+ |
| Parallel | −12126 to −11694 | −51 to −37 | NA | −61 to −59 | −9 to −9 | −3 to −3 | −1 to −1 | 995 to 1101 |
| β-sheet | L− | L− | | s | s | s | s | L+ |
| Antiparallel | −12957 to −12325 | −52 to −51 | NA | −58 to −57 | −9 to −8 | −2 to −2 | −1 to −1 | 1012 to 1166 |
| β-sheet | L− | L− | | s | s | s | s | L+ |
| $^{13}C_{amide}$ | | | | | | | | |
| α-Helix | NA | 14.8 to 16.1 | 182 to 257 | −1080 to −867 | −64 to −29 | 3 to 41 | 74 to 83 | −1353 to −518 |
| | | s+ | L+ | L− | s | s | M+ | L− |
| $3_{10}$-Helix | NA | 11.5 to 16.0 | 275 to 303 | −1131 to −1188 | 0 to 25 | 78 to 91 | 24 to 26 | −1773 to −1271 |
| | | s+ | L+ | L− | s | M+ | s | L− |
| Parallel | NA | 14.3 to 24.4 | 464 to 468 | 260 to 595 | 78 to 88 | 13 to 15 | 5 to 5 | −2023 to −1840 |
| β-sheet | | s+ | L+ | L+ | M+ | s | s | L− |
| Antiparallel | NA | 21.0 to 35.6 | 444 to 454 | 481 to 874 | 69 to 79 | 12 to 13 | 4 to 5 | −2127 to −1913 |
| β-sheet | | s+ | L+ | L+ | M+ | s | s | L− |
| $^{13}C_\alpha$: | | | | | | | | |
| α-Helix | 4602 to 11984 | NE‖ | 91 to 474 | −169 to −133 | −15 to 1 | 17 to 38 | 38 to 42 | NA |
| | L+ | | L+ | M− | s | s | s | |
| $3_{10}$-Helix | 12015 to 15453 | NE | 483 to 692 | −127 to −95 | 20 to 33 | 43 to 47 | 12 to 13 | NA |
| | L+ | | L+ | M− | s | s | s | |
| Parallel | 21904 to 22202 | NE | 995 to 1101 | 217 to 262 | 36 to 40 | 9 to 9 | 4 to 4 | NA |
| β-sheet | L+ | | L+ | L+ | s | s | s | |
| Antiparallel | 21754 to 22655 | NE | 1012 to 1166 | 237 to 295 | 32 to 37 | 8 to 9 | 3 to 3 | NA |
| β-sheet | L+ | | L+ | L+ | s | s | s | |

Peptides are aligned with the bilayer normal. Each range gives the range of CSA or dipolar couplings defined by the largest and smallest of the three measurements made in each case. For example, the range of 86 to 105 PPM reported for the α-helical $^{15}$N CSAs arose from the following three predicted values: Val-12, 105 PPM; Ala-13, 86 PPM; Val-14, 87 PPM.

*The predicted $^{13}C_\alpha$-$^1H_\alpha$ and $^{15}N_{amide}$-$^1H_{amide}$ dipolar couplings can be converted into $^{13}C_\alpha$-$^2H_\alpha$ and $^{15}N_{amide}$-$^2H_{amide}$ dipolar couplings by dividing the couplings listed by 6.5. These can also be approximately converted into $^2$H quadrupolar couplings by multiplying the listed couplings by −5.5 ($^{13}C_\alpha$-$^1H_\alpha$) or by 14 ($^{15}N_{amide}$-$^1H_{amide}$).

‡The +0, +1, +2, +3, and +4 represent the sequence location of the specified isotope relative to residue 12, 13, or 14 (on the amino-terminal side of the

perpendicular to the long axis of the sheet. In these studies, the $z$ computational axis was always assigned to lie in the direction of the bilayer normal.

## Data simulations

A C program "BANDMODS" (Back-calculation of Anisotropic NMR Data from MOlecular Dynamics Simulations) was written to calculate CSA, quadrupolar, and dipolar couplings from either single static structures or ensembles of interconverting structures (such as might generated by molecular dynamics calculations). For the simulations of this study, the bilayer normal was assumed to be aligned with the magnetic field of the spectrometer. No attempt was made to account for local mobility or whole-body motions in the simulations, although these factors are considered at the end of Results. CSAs were predicted using the expression:

$$\mathrm{CSA} = \Delta\delta_{an} = \cos^2\theta_1 \cdot \sigma_1 + \cos^2\theta_2 \cdot \sigma_2 + \cos^2\theta_3 \cdot \sigma_3 - \tfrac{1}{3}(\sigma_1 + \sigma_2 + \sigma_3), \quad (1)$$

where $\sigma_n$ represent the static CSA tensor eigenvalues and $\theta_n$ are the angles made by the nth static tensor eigenvector with respect to the magnetic field, assuming molecular rigidity.

Peptide amide $^{15}$N static tensor eigenvalues and eigenvectors (in the molecular frame) used in the calculations were chosen based upon a rough average of available solutions (Teng and Cross, 1989; Mai et al., 1993; Hartzell et al., 1987). Specifically: $\sigma_1$, 212 parts per million (PPM) (in peptide plane, 15° away from N-H toward the amide bond); $\sigma_2$, 70 PPM (orthogonal to peptide plane); and $\sigma_3$, 47 PPM (in peptide plane). This same model tensor was used in all simulations. The peptide carbonyl $^{13}$C tensor was estimated as an approximate average of known values (Oas et al., 1987; Teng et al., 1992; Stark et al., 1983; Asakawa et al., 1992; Veeman, 1984) to be $\sigma_3$, 95 PPM (orthogonal to peptide plane); $\sigma_2$, 180 PPM (aligned with carbonyl double bond); and $\sigma_1$, 240 PPM (in the peptide plane). For real molecules, both $^{15}$N and $^{13}$C (below) static tensors for peptides vary considerably as a function of local sequence, conformation, and environment (cf. Mai et al. (1993), Teng et al. (1992), and Asakawa et al. (1992)). Thus, even neglecting the many other assumptions inherent in the simulations of this study, the predicted CSA must be considered approximate.

**TABLE 2** *(Continued)*

| $^{13}C_{\alpha}$+1 dipolar | $^{13}C_{\alpha}$+2 dipolar | $^{13}C_{\alpha}$+3 dipolar | $^{13}C_{\alpha}$+4 dipolar | $^{13}C_{amide}$+0 dipolar | $^{13}C_{amide}$+1 dipolar | $^{13}C_{amide}$+2 dipolar | $^{13}C_{amide}$+3 dipolar | $^{13}C_{amide}$+4 dipolar |
|---|---|---|---|---|---|---|---|---|
| −5 to 6 | 8 to 12 | 28 to 34 | 14 to 16 | 182 to 257 | 15 to 24 | 20 to 22 | 21 to 24 | 9 to 10 |
| s | s | s | s | L+ | s | s | s | s |
| 19 to 26 | 26 to 27 | 16 to 17 | 6 to 6 | 275 to 303 | 29 to 34 | 22 to 24 | 11 to 11 | 5 to 5 |
| s | s | s | s | L+ | s | s | s | s |
| 53 to 55 | 13 to 13 | 4 to 5 | 2 to 2 | 464 to 468 | 32 to 32 | 9 to 9 | 3 to 3 | 2 to 2 |
| s | s | s | s | L+ | s | s | s | s |
| 50 to 54 | 10 to 12 | 4 to 4 | 2 to 2 | 444 to 454 | 27 to 31 | 7 to 8 | 3 to 3 | 1 to 1 |
| s | s | s | s | L+ | s | s | s | s |
| 363 to 446 | 19 to 39 | −60 to −43 | −123 to −111 | NA | −33 to 87 | −48 to 0 | −117 to −95 | −63 to −56 |
| L+ | s | s | M− | | s | s | M− | s |
| 343 to 298 | −43 to −27 | −119 to −116 | −34 to −33 | NA | −120 to −51 | −113 to −87 | −70 to −67 | −23 to −22 |
| L+ | s | M− | s | | M− | M− | M− | s |
| −543 to −473 | −93 to −91 | −23 to −21 | −9 to −9 | NA | −306 to −289 | −57 to −55 | −16 to −15 | −7 to −7 |
| L− | M− | s | s | | L− | s | s | s |
| −910 to −538 | −85 to −65 | −20 to −18 | −8 to −7 | NA | −298 to −275 | −53 to −42 | −14 to −13 | −6 to −5 |
| L− | M− | s | s | | L− | s | s | s |
| 43 to 80 | −14 to 30 | −53 to −85 | −60 to −50 | −1353 to −518 | −29 to 5 | −44 to −14 | −79 to −67 | −34 to −29 |
| s | s | s | s | L− | s | s | M− | s |
| 9 to 39 | −58 to −37 | −69 to −67 | −18 to −16 | −1773 to −1271 | −59 to −33 | −74 to −58 | −39 to −38 | −14 to −14 |
| s | s | s | s | L− | s | s | s | s |
| −167 to −146 | −58 to −52 | −15 to −14 | −7 to −7 | −2023 to −1840 | −133 to −129 | −34 to −31 | −11 to −11 | −5 to −5 |
| M− | s | s | s | L− | M− | s | s | s |
| −194 to −156 | −53 to −48 | −14 to −13 | −6 to −6 | −2127 to −1913 | −141 to −124 | −32 to −28 | −11 to −10 | −5 to −5 |
| L− | s | s | s | L− | M− | s | s | s |

latter). For example in the column "$^{15}$N+1" corresponding to the row "$^{13}C_{amide}$ ($\alpha$ helix)" is reported the range spanning the lowest and highest of the following three dipolar couplings: $^{15}$N(Val-12)-$^{13}C_{amide}$(Ala-13), $^{15}$N(Ala-13)-$^{13}C_{amide}$(Val-14), $^{15}$N(Val-14)-$^{13}C_{amide}$(Ile-15).

[§]L, M, and s classify the ranges according to their magnitudes. For dipolar coupling: s, $< |\pm 70|$ Hz; M, |70–150|; L, $> |150|$. For CSA:s; $<|40|$ PPM; L $>|40|$.

[¶]NA, not applicable.

[‖]NE, not estimated.

Dipolar couplings were predicted for pairs of immobilized spin 1/2 nuclei using the following relationship.

$$D_{ij} = \frac{\gamma_i \gamma_j h}{4\pi^2 r^3}(3\cos^2\theta - 1), \quad (2)$$

where the $\gamma$ terms represent the magnetogyric ratios of the interacting nuclei, $r$ is the distance (in Å) between the coupled nuclei, and $\theta$ is the angle between the bilayer normal and the magnetic field. In cases of covalently proximal spins, the possible contribution of scalar coupling was neglected.

## RESULTS

### Simulations of dipolar couplings from membrane proteins under conditions of uniform isotopic labeling

Table 1 lists the numbers and ranges of couplings experienced by single nuclear spins placed near the midpoints of transmembrane domains in the presence of various *uniform* labeling patterns. This table can be approximately extended to the treatment of other possible patterns as described in its footnote.

### Simulations of peptide backbone dipolar couplings and CSAs from isolated transmembrane segments

Simulations most relevant to the NMR elucidation of peptide backbone conformation using specific labeling focussed upon all possible combinations of dipolar couplings between the $\alpha$-$^{13}$C, amide-$^{13}$C, and amide-$^{15}$N of a "target" residue located near the middle of each structure and these same atoms in the four residues each target. While $\alpha$-helices, $3_{10}$-helices, and parallel and antiparallel $\beta$-sheets by no means exhaust the possible secondary structures and conformational motifs of membrane proteins, both sheets and $\alpha$-helices are known to be present in TM domains (Henderson et al., 1990; Deisenhofer and

**TABLE 3 Predicted CSA (PPM) and dipolar couplings (Hz) from the idealized transmembrane peptides (15° tilted)**

| Nucleus on residues 12–14 and 2° structure | $^1$H, directly bonded, dipolar | CSA | $^{15}$N+0 dipolar | $^{15}$N+1 dipolar | $^{15}$N+2 dipolar | $^{15}$N+3 dipolar | $^{15}$N+4 dipolar | $^{13}C_\alpha$+0 dipolar |
|---|---|---|---|---|---|---|---|---|
| $N_{amide}$ | | | | | | | | |
| α-Helix | 15271 to 26375 | 35 to 114 | NA | −8 to 35 | −16 to −1 | −19 to −9 | −9 to −8 | −380 to 1221 |
| $3_{10}$-Helix | 12423 to 26035 | 2 to 66 | NA | −65 to 5 | −25 to −18 | −10 to −10 | −4 to −3 | −248 to 1064 |
| Parallel β-Sheet | −13107 to −6032 | −44 to −37 | NA | −62 to −45 | −8 to −8 | −2 to −2 | −1 to 1 | 414 to 1405 |
| Antiparallel β-Sheet | −12470 to −6838 | −41 to −24 | NA | −55 to −44 | −8 to −7 | −2 to −2 | −1 to −1 | 351 to 1582 |
| $^{13}C_{amide}$ | | | | | | | | |
| α-Helix | NA | 8 to 16 | 16 to 279 | −1211 to −459 | −76 to −10 | −3 to 55 | 72 to 81 | −2273 to 661 |
| $3_{10}$-Helix | NA | 6 to 21 | 161 to 387 | −1218 to −750 | −37 to 91 | 64 to 87 | 17 to 36 | −2729 to 67 |
| Parallel β-Sheet | NA | 13 to 23 | 383 to 452 | −327 to 845 | 67 to 79 | 11 to 13 | 4 to 5 | −3267 to −385 |
| Antiparallel β-Sheet | NA | 17 to 37 | 372 to 436 | −115 to 982 | 60 to 70 | 11 to 12 | 4 to 4 | −3543 to −3135 |
| $^{13}C_\alpha$ | | | | | | | | |
| α-Helix | −7419 to 17009 | NE | −380 to 1221 | −198 to −62 | −26 to 15 | 6 to 40 | 32 to 42 | NA |
| $3_{10}$-Helix | 3688 to 21287 | NE | −248 to 1064 | −184 to −40 | −2 to 51 | 37 to 46 | 8 to 13 | NA |
| Parallel β-Sheet | 13437 to 21611 | NE | 414 to 1405 | 185 to 238 | 31 to 38 | 8 to 8 | 3 to 3 | NA |
| Antiparallel β-Sheet | 14355 to 22047 | NE | 352 to 1582 | 185 to 290 | 28 to 36 | 7 to 8 | 3 to 3 | NA |

Peptides are tilted 15 degrees from the bilayer normal. See footnotes of Table 2 for additional explanation.

Michel, 1991; Weiss and Schultz, 1991), and it has been suggested that $3_{10}$-helices also may be represented (Singer, 1990). It is hoped that simulations for these four structures may provide some insight into the conformation-dependent "dynamic range" of the various spectral parameters.

Because of the uniaxial molecular symmetry and the conformational periodicity within each of the four types of secondary structure examined, it might be tempting to assume that the tensor/vector orientations associated with each class of interaction from *residue* to *residue* would be equal (e.g., all $^{15}N_{amide}$-$^1H_{amide}$ dipolar couplings should be similar for all residues of an α-helix). However, even for a conformationally ideal peptide, this is only the case when the axis of molecular symmetry is aligned with the experimental director; in other words, when helices and sheets are not tilted with respect to the bilayer normal.[3,4] Furthermore, even for untilted helices, internuclear vector/CSA tensor orientations will vary somewhat from residue to residue as a result of local sequence-induced perturbations of idealized molecular geometry. In order to probe the impact of these effects upon dipolar coupling and CSA data, all measurements were repeated for *three* consecutive "target" residues: Val-12, Ala-13, and Val-14 of the TM peptide.

These simulations were carried out for each type of secondary structure at tilt angles of 0°, 2°, 5°, 15°, and 30°. Results for 0° are reported in Table 2. The data are listed as ranges between the lowest and highest of the values predicted for the three residues. It can be observed that the three residues usually yield fairly similar values for each class of data, with the greatest variation occurring for dipolar couplings involving highly proximal spins.[5]

Results from the 2°- and 5°-tilted structures (not shown) are very similar to those of the untilted structure. The results for 15° tilt are presented in Table 3. While results from the 0° structure remain mirrored in many cases (particularly those involving dipolar coupling between relatively distant residues[6]), the three residues sometimes yield widely varying

[3] Technically, the lab frame is defined by the direction of the magnetic field. However, in OSNMR studies involving bilayers, the effective lab frame or "director" axis can usually be defined to be the bilayer normal (see section of Results on dynamics).

[4] This is fairly easy to visualize by considering the carbon-nitrogen dipolar couplings for two directly bound $^{13}C_\alpha$-$^{15}N_{amide}$ pairs on residues $N$ and $N+1$ on an the α-helix. In the untilted peptide the bond vectors (which in this case define the dipolar tensors) will have nearly identical orientations with respect to the bilayer normal (which is the principal axis of the effective lab frame). Now, if the peptide is tilted using the amide carbon-nitrogen bond of residue $N$ as the rotation axis, the orientation of the $^{13}C_\alpha$-$^{15}N$ internuclear vector of residue $N$ with respect to the bilayer normal will not change, unlike that of the corresponding vector in $N+1$.

[5] This latter observation is not surprising. Consider a dipolar coupled spin aligned with the magnetic field. If they separated by 2 Å, a horizontal displacement of one atom by 0.5 Å (due to a minor conformational variation) will result in a 14° change in vector orientation. On the other hand, a 2-Å displacement of one atom of a pair separated by 10 Å, will result in a smaller variation in vector orientation away from 0° (11°), even though the causative conformational or whole-body orientational change may be quite dramatic.

[6] Davis (1988) reported $^2$H spectra of very high resolution from gramicidin reconstituted into magnetically oriented bilayer-like assemblies composed

**TABLE 3** *(Continued)*

| $^{13}C_{\alpha}$+1 dipolar | $^{13}C_{\alpha}$+2 dipolar | $^{13}C_{\alpha}$+3 dipolar | $^{13}C_{\alpha}$+4 dipolar | $^{13}C_{amide}$+0 dipolar | $^{13}C_{amide}$+1 dipolar | $^{13}C_{amide}$+2 dipolar | $^{13}C_{amide}$+3 dipolar | $^{13}C_{amide}$+4 dipolar |
|---|---|---|---|---|---|---|---|---|
| −24 to 30 | −1 to 23 | 23 to 38 | 9 to 17 | 16 to 179 | −1 to 45 | 9 to 27 | 18 to 22 | 7 to 12 |
| −9 to 47 | 16 to 35 | 13 to 17 | 4 to 7 | 161 to 387 | 11 to 39 | 18 to 25 | 8 to 11 | 3 to 5 |
| 48 to 49 | 11 to 13 | 4 to 4 | 2 to 2 | 383 to 452 | 25 to 32 | 7 to 8 | 3 to 3 | 1 to 2 |
| 44 to 49 | 9 to 10 | 3 to 4 | 1 to 2 | 373 to 436 | 25 to 29 | 6 to 8 | 2 to 3 | 1 to 1 |
| 78 to 499 | −15 to 70 | −127 to −13 | −112 to −85 | NA | −110 to 187 | −67 to 23 | −127 to −86 | −64 to −48 |
| 149 to 490 | −111 to 18 | −126 to −86 | −37 to −20 | NA | −230 to 94 | −133 to −55 | −65 to −58 | −25 to −15 |
| −512 to −402 | −93 to −72 | −21 to −20 | −8 to −8 | NA | −329 to −195 | −51 to −47 | −15 to −13 | −6 to −6 |
| −809 to −461 | −65 to −54 | −18 to −16 | −7 to −6 | NA | −302 to −203 | −47 to −43 | −13 to −12 | −6 to −5 |
| −16 to 118 | −28 to 29 | −103 to −37 | −57 to −40 | −2273 to 661 | −59 to 43 | −55 to 5 | −79 to −53 | −35 to −23 |
| −32 to 96 | −78 to −20 | −65 to −59 | −19 to −10 | −2729 to 67 | −80 to 18 | −80 to −49 | −40 to −29 | −16 to −9 |
| −177 to −103 | −51 to −46 | −14 to −12 | −6 to −6 | −3267 to −385 | −119 to −111 | −32 to −27 | −10 to −10 | −5 to −4 |
| −215 to −100 | −47 to −43 | −13 to −11 | −6 to −5 | −3543 to −3135 | −127 to −115 | −30 to −24 | −10 to −9 | −5 to −4 |

values for a given data type (e.g., $^{13}C_{\alpha}$-$^{1}H$ dipolar coupling for $\alpha$-helices).

## Simulation of intersegmental dipolar couplings from a polytopic TM protein

Bacteriorhodopsin (Henderson et al., 1990) was the model employed in these simulations. Dipolar couplings were estimated between the *backbone* carbons and nitrogens of six residues located on helix 1 of BR (Thr-17, Met-20, Gly-23, Thr-24, Phe-27, Gly-31) and all backbone carbons and nitrogens located on the juxtaposed helix 2. Residues on helix 1 were chosen based on the fact that they face helix 2. Over 200 $^{13}C$-$^{13}C$ and $^{13}C$-$^{15}N$ dipolar couplings were estimated (not listed) with the largest observed coupling being 51 Hz. This is rather small, suggesting measurement of dipolar couplings between backbone residues on adjacent TM segments will be very difficult to measure using OSNMR (see Discussion). A search was therefore made for a class of coupling likely to give rise to larger values.

Using interactive graphics, several proximal amino acid pairs were chosen representing juxtaposed helices. For these pairs of residues, the nearest interhelical carbon-carbon distance was recorded, and all possible intraresidue side-chain $^{13}C$-$^{13}C$ dipolar couplings were calculated. These results are summarized in Table 4.

Dipolar coupling between backbone carbons of juxtaposed strands of $\beta$-sheets was also considered. The nearest carbon-carbon distances between two hydrogen-bonded sheets will be in the 3.5–5 Å range. For parallel sheets, $C_{\alpha}$ on one strand will be closest to $^{13}C{=}O$ on another, while for antiparallel sheets the most proximal pair will be $C_{\alpha}/C_{\alpha}$. While no calculations were explicitly made, it can be estimated that for ideal geometries (sheet orientations which would align the internuclear vector with the bilayer normal), the maximum $^{13}C$-$^{13}C$ intrastrand coupling would fall in the range of −250 ± 100 Hz.

## Motional considerations in the interpretation of the results

Rapid whole body or local motions in membrane proteins which result in time-dependent reorientation of the CSA or dipolar tensors will lead to averaging of the $\theta$-dependent parts of Eqs. 1 and 2 over all orientations sampled. In the case of dipolar coupling, rapid variations in $r$ also lead to averaging. "Rapid" motions are those which are fast relative to the frequency ranges spanned by the static dipolar and CSA tensors: typically $>10^4$-$10^5$ $s^{-1}$.

Most membrane proteins execute rapid axial rotation about the bilayer normal (Gennis, 1989; Cherry, 1992) in

unpublished results) that some TM proteins can be successfully be reconstituted into the available magnetically orientable bilayer systems, while other proteins cannot. In these preliminary studies, unlabeled peptides were used and it was not possible to ascertain resonance linewidths from those peptides which were successfully reconstituted.

**TABLE 4 Estimated $^{13}C$-$^{13}C$ dipolar couplings between residue side chains on juxtaposed TM helices of BR and strands of ideal sheets**

| Juxtaposed residue pairs | Nearest interresidue $^{13}C$-$^{13}C$ distance | Number of couplings | Range of interresidue coupling constants* |
|---|---|---|---|
| | Å | | Hz |
| Thr-17-Leu-58 | 3.5 | 8 | 29 to 92 |
| Phe-42-Leu-100 | 3.6 | 28 | −24 to 141 |
| Phe-42-Leu-99 | 3.9 | 28 | −35 to 85 |
| Leu-13-Met-60 | 3.4 | 12 | 24 to 133 |
| Met-56-Ala-84 | 4.1 | 3 | 28 to 112 |
| Leu-48-Leu-92 | 3.2 | 16 | −59 to 156 |
| Val-49-Leu-93 | 3.4 | 12 | 30 to 124 |
| Ile-45-Leu-95 | 4.4 | 16 | 14 to 84 |
| Parallel β-sheet: nearest $^{13}C_\alpha/^{13}C{=}O$ from juxtaposed strands‡ | 3.5–5 Å | 1 | −250 ± 150 |
| Antiparallel β-sheet: nearest $C_\alpha/C_\alpha$ from juxtaposed strands‡ | 3.5–5 Å | 1 | −250 ± 150 |

* These ranges may also be used to estimate couplings between other possible isotopic pairs located on adjacent side chains (see Eq. 2). For examples, these ranges can be converted into $^{15}N$-$^{15}N$, $^{15}N$-$^{13}C$, $^{15}N$-$^{19}F$, $^{19}F$-$^{13}C$, and $^{19}F$-$^{19}F$ couplings by multiplying by 0.16, −0.40, −1.5, 3.7, and 14.

‡ Estimate is made for sheet orientations which would align the relevant internuclear vector with the bilayer normal (see Results).

$L_\alpha$-phase lamellae, but not in the gel phase. In the former case, the splittings predicted by Eqs. 1 and 2 must be scaled down by a factor of $(3\cos^2\alpha - 1)/2$, where $\alpha$ is the angle between the bilayer normal and the magnetic field. This factor is 1.0 for bilayers oriented with normals aligned with the field and −0.5 for orthogonally oriented assemblies. Rapid axial rotation also dictates that Eqs. 1 and 2 must also be redefined so that $\theta$ becomes the angles between the tensor elements and the motional director (the bilayer normal) rather than the magnetic field.

Whole molecule wobbling and local flexibility will also lead to averaging of anisotropic NMR parameters from TM protein domains. For nuclei located on peptide backbones deep within bilayers, available data (Hemminga et al., 1992; Killian et al. 1992; Pauls et al., 1985; Nicholson et al., 1991; Lewis et al., 1985; Herzfeld et al., 1987; Leo et al., 1987) suggests such motions may generally be modest, leading to reductions of anisotropic parameters by only 0–20%. In general, local motions should tend to become more of a factor in regions of the proteins near and in the aqueous phase where water-amide hydrogen bonding can transiently compete with intramolecular hydrogen bonding (see Sami and Dempsey (1988)). However, the available data is rather sparse. The preponderance of Gly and Pro in many TM domains (Williams and Deber, 1991; Li and Deber, 1992) and the general importance of both global and local dynamics in the functions of proteins suggest the backbone dynamics of each protein must be considered individually.

In the case of interresidue side chain $^{13}C$-$^{13}C$ couplings (Table 4), side chain dynamics (e.g., Smith and Oldfield, 1984; Leo et al., 1987) will also result in reductions of coupling constants due to additional averaging of the angular term of Eq. 2. In such instances, the degree of reduction will be highly dependent upon the nature of the specific motions executed.

## DISCUSSION

### Limits of spectral resolution

Practical interpretation of the simulated data set is highly dependent upon the NMR spectral resolution to be anticipated from TM proteins in oriented samples. Currently available data is limited to spectra from proteins in *mechanically* oriented bilayers, where linewidths on the order of 100 Hz have been observed (Teng et al., 1991; Nicholson et al., 1991). While the use of magnetically oriented bilayers may permit spectra of higher resolution to be obtained[7] due to the superior apparent orientational homogeneity of such samples (see Sanders (1993)), this discussion shall conservatively assume the minimum line width from a peptide *backbone* resonance to be 100 Hz. For doublets composed of components 100 Hz wide, interpretive methods based on numerical analysis, spectral simulations, or resolution-enhancement may allow couplings on the order of 50 Hz to be measured with confidence. Based upon these considerations, this discussion conservatively assumes that simulated couplings greater than 70 Hz should be experimentally determinable by OSNMR. It should be noted that rapid sample spinning techniques may afford access to couplings much smaller than this (Smith and Peersen, 1992).

The question of optimizing spectral resolution is relevant to a choice between mechanically or magnetically based methods for orienting bilayers. Certain magnetically oriented bilayer systems have been demonstrated to provide a significantly higher level of spectral resolution than mechanically (glass-plate) oriented bilayers (Sanders, 1993). However, this improvement in resolution is offset by the fact that most magnetically orientable bilayer systems orient with bilayer normals *orthogonal* to the magnetic field. As outlined in the last section of Results, this results in a uniform decrease in the magnitudes of CSAs and dipolar couplings by a factor of two, relative to what would be observed in a 0°-oriented sample (obtainable using the glass-plate method). A spectroscopically ideal situation might arise either from the further improvement of mechanical orientation methods (to reduce the residual line broadening which results from incomplete orientational homogeneity) or from the development of methods for magnetically orienting bilayers such that the bilayer normals are aligned with the field. The feasibility of this latter approach has been demonstrated (Sanders et al., 1993), but

[7] With the advent of homogeneous 17.5 Tesla magnets in NMR spectroscopy, there is hope that these predictions may eventually be proved incorrect, due to the superior resolution and sensitivity which spectrometers equipped with such magnets may afford.

has yet to be optimized in terms of orientational homogeneity and biochemical acceptability.

## What challenges face uniform labeling strategies?

All uniform labeling strategies suffer from the fact that spectral assignments are much more difficult to make in oriented sample NMR than in corresponding solution NMR studies. This is the result of the "through-space" nature of dipolar coupling which dictates that there is no formal relationship between chemical bonding/connectivity and the observation of dipolar coupling between any given pair of spins. However, the assignment problem is probably not insurmountable: even at this early stage, general spectral assignment strategies for oriented sample NMR have been proposed (Sanders, 1993).

A more serious difficulty associated with uniform labeling is exemplified for a fully protonated protein in Table 1. A target proton on an isolated TM helix exhibits significant dipolar coupling to more than 150 other protons. The presence of a second, juxtaposed helix is seen to introduce another 100 significant couplings. While perdeuteration at a very high level (>90%) of enrichment may allow considerable spectral simplification (McDermott et al., 1992; Power and Wasylishen, 1991), the populations of specific multispin systems will become very low, such that sensitivity problems may become severe if measurement of dipolar couplings is sought. Uniform carbon labeling schemes appear to suffer from the same basic complications as $^{1}H$ (Table 1). While the number of coupled nuclei to be reckoned with is reduced, this improvement may be negated by the reduced NMR sensitivity of $^{13}C$, making strategies based on lowering the level of isotopic enrichment difficult.[7]

An additional note should be made at this point regarding the measurement of $X$-$^{1}H$ dipolar couplings between heteronuclei and *directly bonded* protons. Because of the $r^{-3}$ dependency of dipolar coupling (Eq. 2), such couplings are usually much larger than all other $X$-$^{1}H$ dipolar couplings. As a result, even in the presence of considerable broadening due to unresolved long range $X$-$^{1}H$ couplings, the components of the "1-bond" $^{1}H$-$X$ dipolar couplings may often be resolved in the spectrum of the $X$ nucleus (e.g., Sanders and Prestegard, 1991). This is why such couplings were simulated (Tables 2 and 3).

## Specific labeling and pattern recognition

Anticipated difficulties summarized above for uniformly labeled samples can be overcome if *specific labeling* is employed, where emphasis is placed upon the measurement of couplings between a *limited* number of spins. By this route, the number of couplings can be managed and spectral assignments can more easily be made.

An important consideration in choosing a particular labeling strategy is the need to optimize the yield of structural information from a limited number of measurements. Given that the TM domains of most membrane proteins are likely to be dominated by helices or (more rarely) β-sheets (Henderson, 1990; Deisenhofer and Michel, 1991; Weiss and Schulz, 1992), a conceptually attractive labeling strategy would lead to the identification of a particular type of local secondary structure from *patterns* observed in a limited set of data. Pattern recognition plays an important role in solution NMR studies of proteins (Wuthrich, 1986).

The classification of the simulated CSAs and dipolar couplings of Table 2 into various classes based on magnitudes (see Table 2) suggests that patterns do exist which may, in a limited number of cases, be used to provide a preliminary identification of the secondary structure of labeled TM segments based on a few measurements made for that segment. Of course, use of Table 2 in interpreting data from an actual protein of unknown structure would have to be very cautious, taking the possibilities of dynamics and conformations not represented in Table 2 into account.[8] A more realistic use of the "patterns" of Table 2 would be to assist experimental design by identifying specific labeling schemes having a relative high *probability* of providing measurable and structurally useful couplings and CSAs.

Data simulated for structures at various tilt angles (e.g., Table 3) suggest that the classification of parameters according to magnitudes shown in Table 2 no longer generally pertains when the TM segment is tilted by more that about 10° from the bilayer normal. This results from the fact that, while conformation (as defined by a set of dihedral angles) remains fairly constant and essentially *periodic* within a segment of uniform secondary structure, the orientations of nuclear tensors and internuclear vectors with respect to the effective lab frame axis (i.e., usually the bilayer normal) vary *aperiodically* with helix or sheet tilt away from 0°.[9] Tilts of >10° are frequently encountered in the structures of polytopic membrane proteins (Henderson et al., 1990; Weiss and Schulz, 1992; Deisenhofer and Michel, 1991).

## Long-range data constraints for membrane proteins

The possibility of measuring dipolar couplings between nuclei located on residues of adjacent TM segments appears to offer a means of defining the spatial dispositions of adjacent segments, an important step in the determination of tertiary structure. Labeling schemes which would permit measurement *and* assignment of specific $^{1}H$-$^{1}H$ or $^{1}H$-$^{13}C$ dipolar couplings may prove difficult to implement (previous discussion). The simulations suggested that *backbone-backbone* $^{13}C$-$^{13}C$ couplings may be very difficult to resolve by OSNMR, even with very specific labeling (see Results).

[8] For example, pattern recognition may be used as a tool in formulating structural models which are subsequently tested or refined using the more quantitative analytical methods available (see Introduction).

[9] Of course, if the tilted TM segment is undergoing rapid axial rotation about its long molecular axis, periodicity of the tensor orientations with respect to the effective lab frame will be regained.

As illustrated in Table 4, a promising class of intersegment SSNMR data are $^{13}C$-$^{13}C$ dipolar couplings between carbons of adjacent side chains. Based upon the use of specific labeling involving amino acids which are currently commercially available (e.g., *S*-[*methyl*-$^{13}C$]methionine), it is possible to envision schemes which would lead both to unambiguous resonance assignments and to coupling constant measurements. However, as noted at the end of the Results, Table 4 does not take into account the possibility of motional flexibility which would tend to reduce the magnitudes of such couplings (potentially to magnitudes so small that they would be unresolvable). Of course, it is possible that the reduction in couplings resulting from side chain dynamics may also be mirrored and *offset* by reduced linewidths (resolution enhancing) as a result of motion-induced increases in the transverse relaxation times.

The simulations reported in Table 4 suggest an additional set of routes to long range structural constraints. Because $^{3}H$ and $^{19}F$ have magnetogyric ratios roughly four times as large as $^{13}C$, measurement of $^{19}F$-$^{13}C$ or $^{3}H$-$^{13}C$ dipolar couplings should be possible over considerably longer distances than for $^{13}C$-$^{13}C$ interactions. This distance limit would be even further relaxed if experiments could be designed with the goal of measuring $^{19}F$-$^{19}F$ or $^{3}H$-$^{3}H$ couplings. The main drawbacks to these routes are the potential artifacts that could conceivably result from introducing an $^{19}F$ probe (see Ho et al., 1985) into a protein and the difficulty of producing NMR-sufficient quantities of tritium-labeled proteins.

While the simulations of this paper suggest that "long-range" structural constraints for polytopic membrane proteins may indeed be gleaned from oriented sample NMR studies, it should not be overlooked that this is an area in which sample spinning techniques such as REDOR or rotational resonance may prove especially powerful (cf. Smith and Peersen (1992)). Along these same lines, the potential of interfacing solution and solid state NMR technologies in studies of membrane protein structure should not be overlooked (e.g., Shon et al. (1991)).

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