# NMR Measurement of Dipolar Couplings in Proteins Aligned by Transient Binding to Purple Membrane Fragments

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One-bond internuclear dipolar couplings in molecules that are aligned relative to an external magnetic field constrain the orientations of the corresponding vectors relative to the molecule's alignment frame.[1−3] Such couplings can be measured easily if the molecular alignment is very weak, such that only the large one-bond effects are observed as a change in the corresponding one-bond *J* coupling, and broadening resulting from more remote interactions is negligible. For biological macromolecules such as proteins and nucleic acids, the required small degree of alignment with the magnetic field sometimes can be obtained as a result of their own magnetic susceptibility anisotropy,[2,3] or more generally, it can be induced by dissolving them in an anisotropic medium.[1] Lyotropic liquid crystalline media, consisting of planar phospholipid particles known as bicelles,[4] are particularly useful for this purpose.[5] Very recently, it has been demonstrated that nematic phases of rod-shaped virus particles can also be used for obtaining a tunable degree of solute alignment.[6,7] In both cases solute alignment is contingent upon arrangement of individual particles (bicelles or viruses) in a liquid crystalline phase, which collapses below a given threshold concentration. Therefore, there is a lower limit for the degree of solute alignment which can be obtained in such media. Also, there are a number of proteins which destructively interfere with the bicelle liquid crystalline phase and for which dipolar couplings in such media cannot be measured.[5,6] Similarly, it is likely that certain systems will prove unsuitable for measurement in the virus-based media. If a macromolecule can be studied in two or more different oriented media, which in general yield different macromolecular alignment tensors, this dramatically increases the structural information contained in the dipolar couplings.[8]

Here, we demonstrate that a suspension of planar purple membrane (PM) fragments, containing bacteriorhodopsin (BR), can be used to yield the required weak degree of macromolecular alignment in a strong magnetic field. The magnetic susceptibility anisotropy of these PM fragments is dominated by the membrane spanning helices of BR. Their large size (diameter of 0.2 to 2 $\mu$m)[9] and high BR content (75%) result in essentially full alignment of individual particles at field strengths $\geq 10$ T.[9] Unlike the liquid crystalline case, there is no critical lower threshold for their concentration. The PM fragments are highly negatively charged, and the average alignment of two water-soluble proteins is shown to be dominated by electrostatic interactions. The solute alignment tensor obtained in the PM medium, therefore, is expected to be quite different from that in virus- or bicelle-based liquid crystals.

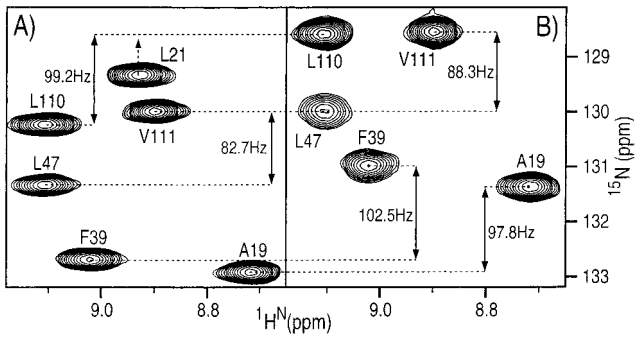


**Figure 1.** Small region of the $^{15}$N−$^{1}$H IPAP-HSQC spectrum[12] of the $^{15}$N-labeled V$\alpha$ domain of the human T-cell receptor (0.4 mM), measured at 600 MHz in 3 mg/mL PM, 93% $H_2O$, 7% $D_2O$, 100 mM NaCl, pH 7.1, 25 °C. Downfield (A) and upfield (B) $^{15}$N−{$^{1}$H$^{N}$} doublet components are shown separately.

Measurement of one-bond dipolar couplings is demonstrated for two $^{15}$N-labeled proteins, the V$\alpha$ domain of the human T-cell receptor, which is one of the proteins which could not be studied in the bicelle medium,[5] and ubiquitin which has been studied extensively by NMR and crystallography.[10,11] Figure 1 shows a small region of the IPAP $^{1}$H−$^{15}$N HSQC spectrum,[12] recorded for 0.4 mM of the V$\alpha$ domain, in a suspension containing 3 mg/mL purple membrane fragments, 100 mM NaCl, pH 7.1. Substantial deviations from the isotropic $^{1}J_{NH}$ splitting (ca. 94 Hz) can be seen, indicating significant alignment. In the frame of the diagonalized molecular alignment tensor, the dipolar couplings can be described by the equation

$$^{1}D_{NH} = D_a^{AB}\{(3\cos^2\theta - 1) + {}^{3}/_{2}R(\sin^2\theta\cos 2\phi)\} \quad (1)$$

where $\theta$ and $\phi$ are the spherical coordinates describing the orientation of the internuclear vector, and $D_a$ and $R$ are related to the magnitude and rhombicity of the alignment tensor, as described elsewhere.[13] A best fit between the observed dipolar couplings and a preliminary NMR structure (Hu, J.-S., unpublished results), calculated without dipolar couplings, shows reasonable agreement with a correlation coefficient $R = 0.90$ (Supporting Information).

The PM fragments have their normal parallel to the magnetic field,[9] whereas the bicelle normal is orthogonal to the field.[4] Therefore, if molecular alignment were caused exclusively by steric interactions between the surface of the idealized flat, smooth surfaces of the fully aligned purple membrane and bicelle surfaces,

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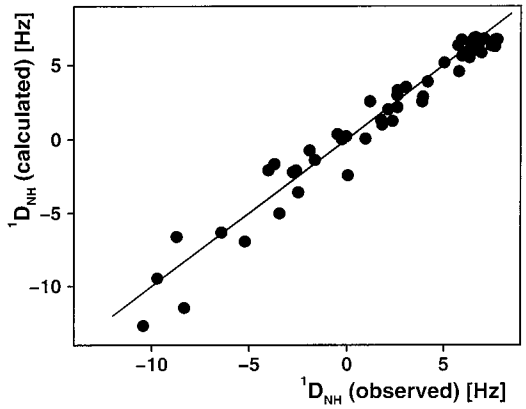


**Figure 2.** $^{15}N-^{1}H$ one-bond dipolar couplings, $^1D_{NH}$, measured for 57 backbone amides in U-$^{15}N$ ubiquitin in 1 mg/mL PM, 50 mM NaCl, pH 6.8, 35 °C, at 600 MHz, versus values calculated for the solution structure, using $D_a = -6.52$; $R = 0.05$; $\alpha = 60°$; $\beta = 150°$; $\gamma = 41°$, where $\alpha$, $\beta$, and $\gamma$ are the Euler angles describing the orientation of the alignment tensor relative to the solution structure, whose orientation has been best fit to that of the crystal structure.[11] Residues from the flexible C-terminus are not included. The solution structure is based on 2727 NOEs, 94 torsion angle restraints, and 940 dipolar couplings measured in neutral and positively charged bicelle media (Marquardt, J. L., unpublished).

the dipolar couplings observed in the purple membrane suspension would be scaled by a factor −2 relative to the couplings in a bicelle solution (assuming the same flat surface area per unit volume). Based on X-ray diffraction data for PM dispersions[14] and DMPC vesicles,[15] the cross-sectional area per gram of PM is about 25% smaller than for the bicelle DMPC bilayer. Therefore, if molecular alignment were entirely due to steric effects, solute alignment in PM suspensions would be expected to be about $2 \times 0.75 = 1.5$ times larger than in the bicelle medium, for a given weight per volume ratio. Instead, the value for the Vα alignment tensor in the very dilute purple membrane suspension is unusually strong compared to alignments of proteins observed in much more concentrated bicelle solutions.[5] This suggests that weak electrostatic interactions between the Vα domain and the strongly negatively charged PM surface play a significant role in causing the alignment.

To investigate whether the transient binding of a protein to PM affects its structure, we also have measured $^1D_{NH}$ dipolar couplings in U-$^{15}N$ ubiquitin, dissolved in this medium. The protein (0.5 mM) was dissolved in a suspension of 1 mg PM/mL, 50 mM NaCl. Addition of NaCl was necessary to weaken the electrostatic interaction between ubiquitin and PM; below 50 mM salt, the binding of ubiquitin to PM becomes too strong and results in considerable line broadening. At higher salt, ubiquitin alignment decreases, and $^{15}N$ $T_2$ values increase and approach those measured in isotropic solution. Figure 2 shows good agreement between $^1D_{NH}$ splittings measured in the PM medium and values calculated from a best fit to the solution structure.[10] When the $^1D_{NH}$ values are compared with the X-ray structure (Supporting Information), the only two outliers in this correlation (Arg[54] and Val[70]) correspond to residues which have slightly different N−H bond orientations in the crystal and previously calculated solution structures. This suggests that, although the solution and crystal structures of ubiquitin are very similar, the PM-bound conformation is closest to the solution structure. The orientation of the alignment tensor of ubiquitin in PM medium is very different from that in bicelles[5,8] (Supporting Information), and the dipolar couplings in PM provide important complementary information.[8]

PM fragments are easily isolated and purified in large quantities from the cell membrane of *Halobacterium salinarium*.[16] Suspensions remain stable for many months when kept at low ionic strength. However, high salt concentrations weaken the electrostatic repulsion between PM fragments and noncoplanar aggregation of the PM fragments ("clumping") can occur, which decreases the net solute alignment. As with bicelles, the $^2H$ quadrupole splitting, $\Delta\nu_Q$, of solvent deuterons can be used to monitor alignment of PM in the magnetic field. For 9 mg of PM per mL of distilled water (90/10 $H_2O/D_2O$), $\Delta\nu_Q$ is 2.6 Hz and remains stable for at least one month. However, if 50 mM NaCl is added, $\Delta\nu_Q$ decreases rapidly due to clumping, and no splitting is observed after 15 h if the sample is kept outside the magnet (Supporting Information). In contrast, if the sample is placed inside the 14-T NMR magnet immediately after addition of salt, $\Delta\nu_Q$ remains stable and no clumping occurs. Remarkably, after this salt-containing PM sample is taken out of the magnet, clumping occurs much more slowly than in an identical sample which has not been exposed to the magnetic field (Supporting Information).

PM provides a convenient medium for measuring dipolar couplings in macromolecules. Dipolar couplings measured for solutes which transiently bind to the oriented PM reflect the conformation in the PM-bound state. Analogous to the transferred NOE effect,[17] dipolar couplings therefore offer promise to study the conformation of flexible ligands that transiently bind to integral membrane proteins anchored in oriented bilayers.

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**Supporting Information Available:** Plots of measured Vα $^1D_{NH}$ couplings vs those predicted by a preliminary NMR structure; measured ubiquitin $^1D_{NH}$ couplings vs those predicted by the X-ray structure; ubiquitin $^1D_{NH}$ couplings measured in neutral bicelles vs those in PM; plots of the solvent $^2H$ $\Delta\nu_Q$, showing the effect of magnetic field on PM clumping (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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