Communication

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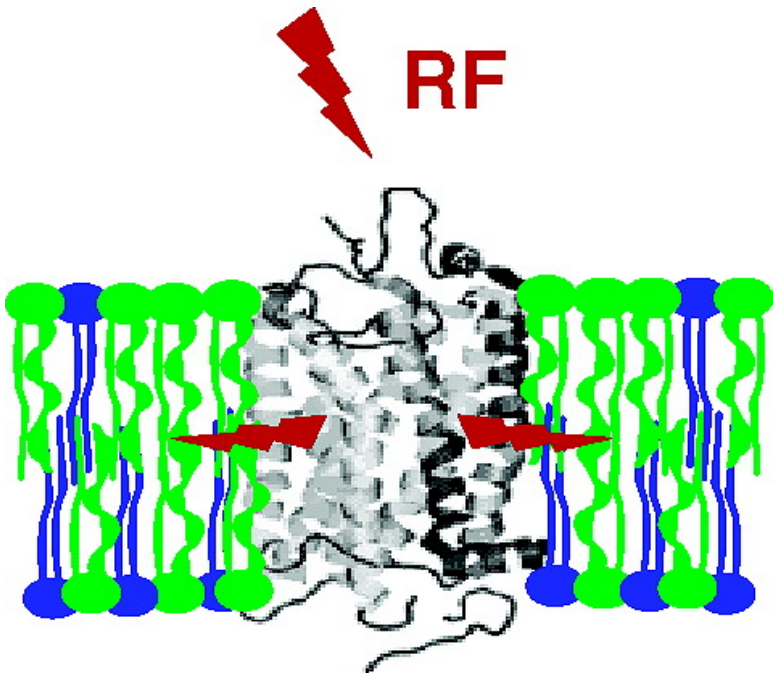

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Probing Specific Lipid−Protein Interaction by Saturation Transfer Difference NMR Spectroscopy

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Recently attention has focused again on the importance of the lipid matrix for function of integral membrane proteins.[1−4] From early EPR and NMR studies it is well-known that lipids in a first layer surrounding integral membrane proteins have different dynamic properties.[5,6] By fluorescence spectroscopy as well as chemical cross-linking, evidence for preferential interactions between particular lipid species and proteins was obtained.[7,8] More recently, the existence of tightly bound lipids in membrane protein crystals was reported.[9] While the importance of those observations is indisputable, such experiments should be conducted in the biologically relevant liquid crystalline state of the lipid matrix without using potentially perturbing labels.

Here we applied saturation transfer difference (STD) spectroscopy in combination with magic angle spinning (MAS) to probe specific lipid−protein interactions in a liquid crystalline lipid matrix. Saturation transfer experiments have a long history[10] and are widely used for the study of protein−ligand interactions by high-resolution NMR.[11−13] We show that STD-MAS NMR on membranes with incorporated proteins reveals the individual strength as well as the statistics of lipid−protein interaction. Figure 1A shows the pulse sequence of the ^{1}H STD-MAS NMR experiment. Membrane protein resonances are selectively saturated via frequency selective radio frequency (rf) pulses (Figure 2). The magnetization redistributes within the protein via spin diffusion. Magnetization is then transferred from the protein to a first layer of lipids surrounding the protein via ^{1}H−^{1}H dipolar contacts. An efficient intermolecular magnetization transfer from protein to lipid requires short distances between protons, equivalent to physical contact between molecules. While saturation energy continues to enter the system through the sustained application of a rf signal, exchange of lipids between the first layer and the surrounding lipid matrix takes place, thus increasing the population of rf-saturated lipid. If the lifetime of lipid−protein interaction is much shorter than the saturation time, then all lipids in the matrix may reach the protein, and the fractional decline of lipid resonance intensity is a measure of the probability for a specific lipid to interact.

We applied this new approach to membranes composed of polyunsaturated 1-stearoyl-d35-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (18:0d35-22:6-PC) and the monounsaturated 1-palmitoyl-d31-2-oleoyl-*sn*-glycero-3-phosphocholine (16:0d31-18:1-PC) and bovine rhodopsin. A typical ^{1}H MAS NMR spectrum of the reconstituted membranes is shown in Figure 1B. The upper spectrum was acquired with the saturation frequency located at 12 ppm, far away from lipid and protein MAS center- and side-bands resonances. The lower spectrum was acquired with the saturation frequency at 8.35 ppm, which corresponds to the region of amide and aromatic amino acid side-chain resonances. The peak at 2.85 ppm is from methylene protons of the docosahexaenoyl acyl chain (DHA), while the resonance at 1.3 ppm is dominated by a resonance of methylene protons of oleic acid (OA) as well as a small

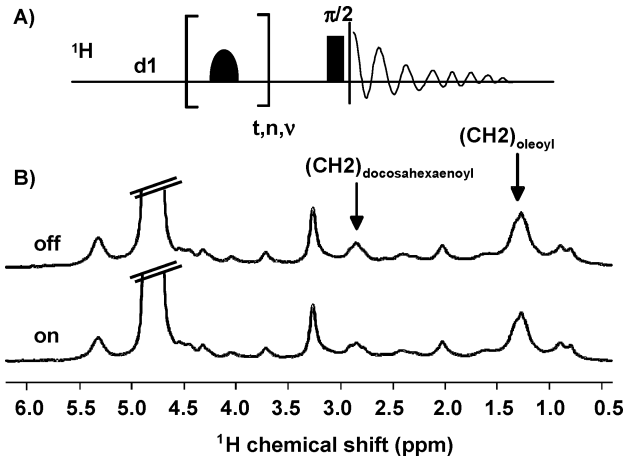

Figure 1. (A) Pulse sequence for saturation transfer difference MAS NMR spectroscopy: t = width of the Gaussian pulse, n = number of repetitions, and ν = saturation frequency. (B) Saturation transfer ^{1}H MAS spectra of membranes (∼0.1 mg) composed of rhodopsin/18:0d35-22:6-PC/16:0d31-18:1-PC, 1/125/125, mol/mol/mol) in 10 mM PIPES, 100 mM NaCl, 50 μM DTPA, in D_2O, pD = 7.4. Proteoliposomes[3,4] were deposited into the pores of anodic aluminum oxide (AAO) disks. Sample preparation and experiments were conducted in complete darkness. The disks in buffer solution were cut with a scalpel into small pieces and packed into the spherical volume of a Kel-F insert inside a 4-mm MAS rotor. The upper spectrum was recorded with saturation at 12 ppm (off resonance) and the lower spectrum at 8.35 ppm (rhodopsin amide/aromatic resonances, on resonance). Experiments were conducted on a Bruker AV800 spectrometer at a resonance frequency of 800.18 MHz. A single Gaussian-shaped pulse of 5-ms length, rf field strength of 6.5 kHz gave optimal conditions for selective saturation of protein resonances without saturating lipids. (MAS frequency 10 kHz; temperature 25 °C; $\pi/2$ pulse length 2.85 μs, 16 scans per spectrum, 10 Hz line broadening).

percentage of intensity from rhodopsin methyl resonances. Magnetization transfer from lipids is seen as a decrease in resonance intensities.

Figure 2 shows the saturation time dependence of DHA and OA resonances of 16:0d31-18:1-PC/rhodopsin and 18:0d35-22:6-PC/rhodopsin membranes as well as of the mixed lipid membrane. The DHA resonance at 2.85 ppm is preferentially attenuated, highlighting a preference for magnetization transfer to the DHA chain. For saturation times from 5 to 25 ms (t = 5 ms, n = 1−5), signal attenuation increased linearly with saturation time. This is in agreement with exchange of lipids between an interaction site on the protein and the bulk matrix on the time scale of milliseconds or faster. Reducing the temperature from 35 to 10 °C increased the rates of magnetization transfer, most likely as a result of reduced molecular motions. This also suggests that the rate-limiting step is magnetization transfer from protein to lipid. If the exchange of lipids between an interaction site of the protein and the bulk matrix was rate limiting, then transfer rates would have decreased with decreasing temperature. The rate of magnetization transfer between

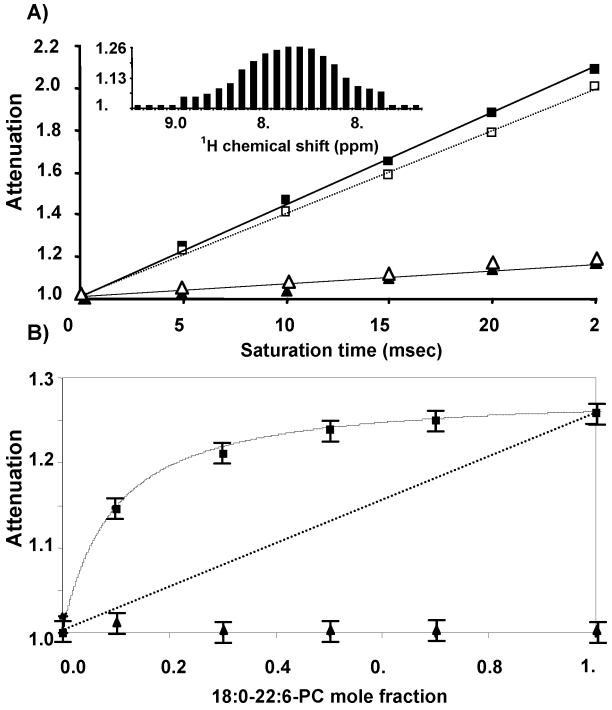

Figure 2. (A) Saturation time dependence of the attenuation factor of oleic (OA, triangles) and of docosahexaenoic (DHA, squares). The attenuation factor is defined as integral signal intensity with 12 ppm (off resonance) saturation divided by the integral signal intensity with 8.38 ppm (on resonance) saturation. Filled symbols correspond to attenuation measured in 16:0d31-18:1-PC/rhodopsin (250/1, mol/mol) and 18:0d35-22:6-PC/rhodopsin (250/1, mol/mol) membranes, open symbols are for a 16:0d31-18:1-PC/18:0d35-22:6-PC/rhodopsin (125/125/1, mol/mol/mol) mixture. Attenuation of the DHA signal as a function of the saturation frequency (expressed in ppm) is shown in the insert to Figure A. (B) Concentration dependence of signal attenuation of DHA (filled squares) and OA (filled triangles) reported as a function of the 18:0-22:6-PC mole fraction in 16:0d31-18:1-PC/18:0d35-22:6-PC mixed membranes keeping the total lipid/protein ratio constant at 250/1. Experiments were conducted at 30 °C.

lipid molecules was measured earlier by NOESY cross-relaxation and deemed to be negligible at the short saturation times of this experiment.[14,15] The frequency dependence of DHA signal attenuation is shown in the insert to Figure 2A. Maximum attenuation was obtained for saturation at 8.35 ppm, corresponding to the chemical shift range of amide- and aromatic side-chain protons. All further experiments were conducted with a 5-ms Gaussian-shaped saturation pulse applied at 8.35 ppm.

The polyunsaturated DHA chains displayed a much higher rate of transfer than the monounsaturated OA chains, indicating that interactions between rhodopsin and DHA chains are significantly stronger. There are indications from the ^{1}H MAS NMR spectra that saturation of rhodopsin side-chain methyl resonances is not as efficient as saturation of amide and C_α backbone resonances. Therefore, rates of magnetization transfer to lipids may differ between interaction sites at the protein. They are likely to be highest for lipid hydrocarbon chain interaction with the backbone of α-helices. Indeed, it had been proposed that polyunsaturated chains may fit neatly to the structure of α-helices[16] which may explain the higher rates of DHA saturation. Furthermore, in 18:0d35-22:6-PC/16:0d31-18:1-PC/rhodopsin mixtures at an equimolar ratio of DHA and OA, rates of magnetization transfer to DHA chains were almost identical to rates in 18:0d35-22:6-PC/rhodopsin membranes. This indicates that the monounsaturated 16:0d31-18:1-PC does not compete efficiently for interaction sites at the surface of rhodopsin, which is confirmed by the plot of DHA resonance attenuation as a function of 16:0d31-18:1-PC molar fraction (Figure 2B). If the polyunsaturated lipid would have replaced the monounsaturated lipid from the surface of rhodopsin proportionally to its concentration in the lipid matrix, a linear increase of DHA resonance attenuation would have resulted (dashed line). Instead DHA resonance attenuation reaches much higher values at lower 18:0d35-22:6-PC concentrations, confirming the strong preference for interaction of the protein with the DHA chain. The dependence of signal attenuation as a function of the 18:0-22:6-PC mole fraction was fitted to a model of competitive binding which yielded a ratio of DHA/OA association constants of 14 ± 2 and a number of binding sites on rhodopsin of 5 ± 4, confirming the large preference of rhodopsin for interaction with DHA.

The preference for rhodopsin interaction with polyunsaturated chains is even detected in an 18:0-22:6-PC/rhodopsin membrane. The resonance of polyunsaturated DHA was attenuated by a factor of 1.25, while the saturated SA resonance showed only very little attenuation (1.05), confirming the dramatic preference for solvation of rhodopsin by DHA chains. The results suggest a radial distribution of lipids with the polyunsaturated chains oriented preferentially toward the transmembrane helices of rhodopsin.[16]

In conclusion, we have shown that the GPCR rhodopsin exhibits a preference for interactions with polyunsaturated docosahexaenoic acid. Results were obtained by STD-MAS NMR, which requires relatively little membrane material (~0.1 mg) making it applicable to model as well as biological membranes. Spectral resolution for magnetization transfer to specific lipid resonances could be further improved by transferring proton magnetization to the ^{13}C resonances of lipids. The much wider distribution of ^{13}C chemical shifts is less likely to result in spectral superposition of resonances. The study of the interaction of ligands with membrane proteins in a lipid matrix by STD-MAS appears to be feasible as well.

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