Evidence for Specificity in Lipid-Rhodopsin Interactions*

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The interaction of bovine rhodopsin with poly- and monounsaturated lipids was studied by \(^1\)H MAS NMR with magnetization transfer from rhodopsin to lipid. Experiments were conducted on bovine rod outer segment (ROS) disks and on recombinant membranes containing lipids with polyunsaturated, docosahexaenoyl (DHA) chains. Poly- and monounsaturated lipids interact specifically with different sites on the rhodopsin surface. Rates of magnetization transfer from protein to DHA are lipid headgroup-dependent and increased in the sequence PC < PS < PE. Boundary lipids are in fast exchange with the lipid matrix on a time scale of milliseconds or shorter. All rhodopsin photointermediates transferred magnetization preferentially to DHA-containing lipids, but highest rates were observed for Meta-III rhodopsin. The experiments show clearly that the surface of rhodopsin has sites for specific interaction with lipids. Current theories of lipid-protein interaction do not account for such surface heterogeneity.

Rhodopsin is the light receptor responsible for dim light vision in the rod photoreceptor cells of vertebrates. A large body of research demonstrated that efficiency of the rhodopsin-dependent steps of the visual process is exquisitely sensitive to membrane lipid composition, in particular to the content of \(\omega-3\) polyunsaturates (1–3). Retinal membranes of mammals, similar to synaptosomal membranes in brain, contain up to 50 mol % of docosahexaenoic acid (DHA, \(^{22}\text{26:3}\)), a polyunsaturated fatty acid with 22 carbon atoms and six double bonds that are evenly distributed over the length of the chain (4).

In earlier reconstitution experiments with bovine rhodopsin it was established that the equilibrium concentration of Meta-II rhodopsin increased with the concentration of DHA hydrocarbon chains in the lipid matrix (5–7). Also the headgroups of phospholipids had a significant effect on Meta-II formation (5, 7) and activation of \(G_t\) (8, 9). It was observed that phosphatidylethanolamines (PE) and the negatively charged phosphatidylserines (PS) increased the amount of Meta-II.

The influence of PS on the equilibrium was assigned to changes of the membrane electric surface potential (7). In contrast, the sensitivity of membranes to PE content correlated with an alteration of membrane curvature elasticity (10) as proposed for other membrane proteins by Navarro et al. (11), Jensen and Schützbach (12), Gruner (13), Lindblom and co-workers (14), and Cantor (15). Litman and co-workers (6, 16) found a correlation between mobility and orientation of the fluorescence probe DPH in lipid bilayers, summarized as a membrane free volume parameter, and Meta-II formation. Furthermore, they observed a preference of rhodopsin to locate in domains rich in di-\(22:6n3\)-PC that formed in di-\(16:0\)/di-\(22:6\)/cholesterol mixtures. Mouritsen proposed a link between hydrophobic thickness of lipid bilayers and activity of membrane proteins (18). Brown and co-workers (10) suggested that Meta-II has a greater hydrophobic thickness than Meta-I, and that the lipid bilayer has to thicken to match the hydrophobic length of Meta-II. They combined hydrophobic mismatch and curvature elasticity into a flexible surface model that links membrane elastic properties to rhodopsin function in a very general way (10).

In this project we explored whether the rhodopsin surface should be viewed as homogeneous and the surrounding membrane as a continuum, or if specific interactions with lipids could play a role in rhodopsin activation. Lipids could associate with particular sites on the protein and alter function, in analogy to lipid-like substances that act as ligands. Such specific interactions are likely to depend on the chemical composition of lipid hydrocarbon chains, in particular polyunsaturation, and on lipid headgroups. Indeed crystal structures of bacteriorhodopsin (19–22) and bovine rhodopsin (23, 24) show a few structurally well defined lipids that interact with rhodopsin helices. Molecular dynamic simulations reported existence of specific sites on the surface of rhodopsin for specific interaction with polyunsaturated chains, saturated chains, and cholesterol (25). While those observations are very important, they should be verified by results from a method that explores such interactions in fluid bilayers at physiologically relevant conditions.

Recently, we demonstrated the feasibility of investigating lipid-rhodopsin interaction by saturation transfer NMR (ST-NMR) in combination with magic angle spinning (MAS) (26). A preliminary account of saturation transfer by selective excitation between the protons of rhodopsin and lipids, without application of MAS, was presented earlier by Deese et al. (27). In ST-NMR, membrane protein resonances are selectively saturated via radiofrequency (rf) pulses. Magnetization is then transferred from the protein to a first layer of lipids surrounding the protein via \(^1\)H–\(^1\)H dipolar contacts allowing identification of the lipid species that are interacting with the protein.
In this study, lipid-rhodopsin interactions were investigated by ST-MAS-NMR. We report the results obtained on natural ROS disks and on reconstituted model membranes. After phototransformation of rhodopsin in the spinning rotor, the photointermediates responsible for lipid resonance attenuation were identified through their temperature and pH dependence as well as their lifetimes. Headgroup selective transfer of magnetization was probed via a transfer of proton magnetization to lipid phosphorus nuclei. In addition, rhodopsin was reconstituted into binary mixtures of poly- and monounsaturated lipids. The experiments revealed that lipids with polyunsaturated docosahexaenoyl and monounsaturated oleoyl chains interact specifically with different sites on the surface of rhodopsin.

**EXPERIMENTAL PROCEDURES**

**Preparation of Native ROS Disks**

Intact disk membranes were isolated from rod outer segments (ROS) of bovine retinas as described elsewhere (28). Samples were kept at 4 °C, and all manipulations were performed in the dark or under dim red light. Before conducting the NMR experiments, the native ROS disks were exchanged into the appropriate D$_2$O buffer (10 mM Tris-HCl, 60 mM KCl, 30 mM NaCl, 2 mM MgCl$_2$, 50 µM DTPA, 1.5 mM dithiothreitol, 1.5 mg/ml aprotinin, pD of 8.4 or 6.4) by several cycles of centrifugation followed by resuspension.

**Preparation of Recombinant Membranes**

The phospholipids 1-perdeutero-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine (18:0d35-22:6n3-PC), 1-perdeutero-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphoethanolamine (18:0d35-22:6n3-PE), 1-perdeutero-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphoserine (18:0d35-22:6n3-PS) and 1-perdeutero-palmityl-2-oleoyl-sn-glycero-3-phosphocholine (16:0d31-18:1n9-PC) were synthesized by Avanti Polar Lipids Inc. (Alabaster, AL). Polyunsaturated lipids were stored at −80 °C in sealed, argon-filled ampoules as solution in methylene chloride or chloroform with the antioxidant butylated hydroxytoluene (BHT) added at a lipid/BHT molar ratio of 200:1. Sample preparation was carried out in a nitrogen-filled glove box to prevent oxidation. Rhodopsin was purified from bovine retinas using procedures that were developed by Litman (28). Rhodopsin fractions in 3 wt. % octylglucoside (OG), which gave a 280/500 nm intensity ratio of 1.9 or better in the uv/vis absorption spectra, were used for reconstitution. To recombine rhodopsin with lipids, the phospholipids in organic solvent were dried in a stream of pure nitrogen gas in a glass round bottom flask. The OG-solubilized rhodopsin was added to the dry lipid to form mixed micelles with an OG/lipid molar ratio of about 10:1 and a phospholipid/rhodopsin molar ratio of 250:1. The micellar solution was then rapidly diluted by injection into PIPES buffer (10 mM PIPES, 100 mM NaCl, 50 µM DTPA, pD 7.4) prepared with D$_2$O, which resulted in the formation of proteoliposomes. Care was taken to ensure that the final OG concentration was not higher than 5 mM, which is well below the cmc of OG. The proteoliposome dispersion (typically 0.5 mg lipids/ml) was then extruded through a stack of two porous anodic aluminum oxide (AAO) filters, 13-mm diameter, with a nominal pore diameter of 0.2 µm (Whatman), resulting in entrapment of multilamellar bilayers inside the pores (29). All but a single, cylindrical bilayer near the AAO pore surface, as well as residual OG in membranes, were removed by forceful flushing of filters with several milliliters of plain buffer at a rate of 0.2 ml/s (29). The top filter was discarded, and the second filter cut into small pieces with a blade. The wet pieces were transferred into an 11-µl spherical insert for MAS rotors made of Kel-F (Bruker Biospin Inc., Billerica MA).

**NMR Experiments**

**ST-NMR on ROS Disks**—The NMR experiments on ROS disks were conducted on a Bruker DMX500 NMR spectrometer equipped with a wide bore 11.7 T magnet. The $^1$H spectra were acquired in a triple resonance $^1$H/$^{13}$C/$^2$H, 4 mm MAS probehead (Bruker Biospin, Inc.) at a MAS spinning frequency of 10 kHz with a $^1$H π/2 pulse length of 4 µs. Typically 4 scans with a recycle delay of 10 s were acquired. A Gaussian-shaped pulse of 50 ms length at an rf field strength of 0.12 kHz gave optimal conditions for selective saturation of protein resonances without saturating lipids. A total saturation length of 500 ms (ten 50-ms Gaussian pulses) was used if not stated otherwise. The resonance frequency of rf-presaturation corresponded to a chemical shift of 8.5 ppm in the proton spectrum. When needed, samples in the spinning MAS rotor were illuminated for 1 min with light from a miniature light bulb.

Please note that the saturation field strength was much lower and the length of the presaturation pulse substantially longer compared with our previously reported experiments (26). Conditions were changed to address a recent observation that magic angle spinning may result in modulation of the saturation frequency such that weak sidebands, different from multiples of the spinning frequency, may occur. Current experimental conditions completely avoid any saturation of lipid resonances that is not transmitted to the lipid matrix from rhodopsin.

The $^{31}$P NMR spectra were acquired with a $^1$H/$^{31}$P-MAS probehead at 10 kHz MAS with a $^3$P π/2 pulse length of 9.5 µs, and a sequence delay time of 5 s. A $^1$H-preservation of 500 ms at a $^1$H B$_1$ field strength of 0.12 kHz was applied before a 10-ms $^3$P $^1$H cross-polarization that was linearly ramped on the proton channel from 25 to 34 kHz (see Fig. 1). To compensate for the temperature increase from rapid sample spinning and/or from application of rf fields, the temperature of the bearing air was lowered with a chiller. The temperature inside the spinning rotor was calibrated by measurement of the temperature-dependent chemical shift of the water resonance as well as by following known phase transitions of lipids.

**ST-NMR on Reconstituted Membranes**—The experiments were carried out on a Bruker AV800 NMR spectrometer, equipped with a 18.8 T standard bore magnet and a $^1$H/$^{13}$C/$^2$H MAS probe at a MAS frequency of 15 kHz and a $^1$H π/2 pulse length of 2.9 µs. The higher MAS frequency necessitated a slightly higher level of rf field strength (1 kHz) for presaturation. Other conditions were identical to the experiments conducted at 11.7 T. Typically 16 transients were acquired for each experiment. Sample temperature was adjusted via a stream of temperature controlled gas directed into the rf coil compart-
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**RESULTS**

**1H ST-MAS NMR on ROS Disks**—Rhodopsin interaction with saturated and unsaturated hydrocarbon chains of phospholipids was investigated in bovine ROS disks. According to published results, the disks are composed of phospholipids containing ~50% polyunsaturated acyl chains (~65% of them DHA) and ~40% saturated chains (stearic and palmitic acid) (30). Rhodopsin constitutes 90–95% of all membrane proteins in ROS disks (31). Fig. 2, A and B show the 1H NMR spectra of ROS disks recorded at a MAS spinning frequency of 10 kHz without (A) and with (B) selective rf saturation of rhodopsin, respectively. The resonance at 2.85 ppm stems from the protons of methylene groups located between the double bonds in polyunsaturated chains (mostly DHA), whereas the resonance at 1.3 ppm is dominated by methylene protons from stearic and palmitic acid with a small contribution from unsaturated fatty acids other than DHA and a minor contribution from methyl groups of rhodopsin. The integral intensity ratio of the 2.85 ppm to 1.3 ppm resonances agrees very well with the reported fatty acid content of ROS.

The saturation time, saturation frequency, and saturation power for ST-NMR were optimized in experiments on ROS disks and on recombinant samples with and without rhodopsin. No attenuation of lipid resonances in protein-free membrane samples was observed at presaturation with a B1 field strength of 0.25 kHz or less, applied at a frequency corresponding to a chemical shift of 8.5 ppm or larger for up to 500 ms. But an attenuation of lipid resonances was observed for membranes containing rhodopsin, even at lower B1 field strength. Lipid resonance attenuation at a B1 field of 0.12 kHz increased linearly with increasing presaturation time, up to 500 ms (see Fig. 2C). A saturation time of 500 ms, a B1 field strength of 0.12 kHz at a frequency corresponding to a chemical shift of 8.5 ppm gave optimal conditions for selective saturation of rhodopsin without directly saturating lipid resonances.

The rf saturation of rhodopsin in ROS resulted in an attenuation of the 2.85 ppm resonance (predominantly DHA) by a factor of 1.66 ± 0.02, whereas the resonance at 1.3 ppm (predominantly saturated chains) was attenuated by a factor of 1.54 ± 0.02 (Fig. 2). Attenuation is defined as intensity ratio of the resonance recorded without and with presaturation. We also established that both resonances had the same spin-lattice relaxation time of 0.95 ± 0.05 s indicating that differences in spin-lattice relaxation are not responsible for the preferential attenuation of the DHA resonance.

31P ST-MAS NMR on ROS—The bovine ROS disk membranes are composed of 40–50 mol % PC, 30–35 mol % PE, 20–30 mol % phosphatidylethanolamine plasmalogens, and 5–10 mol % PS (32). The 31P MAS NMR spectrum recorded with a hard 31P π/2-pulse and a long delay time between scans of 5 s, to ensure proportionality of signal intensity to the molar ratio of phospholipid species, is shown in Fig. 3A. Magic angle spinning averages the powder pattern from the 31P NMR anisotropy of chemical shift to a rotational centerband and sidebands that are separated from the centerband by multiples of the MAS spinning frequency. In Fig. 3 only the MAS centerband is shown. At 10 kHz spinning frequency, sideband intensities can be safely ignored, and the intensities of PC, PE, and PS centerband resonances reflect composition of the ROS. Resonances were assigned by comparison with spectra of model membranes with known composition (see also Ref. 33). The fit to the experimental spectrum (Fig. 3B) yielded a PE/PC/PS molar ratio of 4.2:4.8:1. Considering that ethanolamine plasmalogens and PE are likely to have the same 31P NMR chemical shift, this molar ratio is in good agreement with expectations.

Rhodopsin interaction with specific lipid species was investigated by ST-MAS NMR with 1H-31P cross-polarization (CP). After presaturation of rhodopsin, CP transfer occurs via intramolecular dipolar couplings from the protons of lipid glycerol groups and headgroups to the lipid phosphorus nuclei as well as via intermolecular 1H-31P dipolar couplings between...
rhodopsin protons and lipid phosphorus. It was previously reported that the methylene protons of g3, and of the α-methylene group of headgroups are the primary contributors to intramolecular dipolar interaction with the $^{31}$P nucleus (34, 35). During the long saturation time of 500 ms, magnetization is partially exchanged by spin-diffusion within the intramolecular proton network of lipids (36). Therefore, the attenuation of the phosphorus resonance is likely to reflect interactions of all lipid segments with sites on rhodopsin. Fig. 3C shows the $^{31}$P CP-MAS-NMR spectra obtained without (solid line) and with (dashed line) presaturation of rhodopsin protons. As can be seen, both PC and PE resonances are attenuated when rhodopsin protons are presaturated. This indicates that both phospholipids are interacting with rhodopsin. However, the $^{31}$P resonance of PE is more attenuated than the resonance of PC (attenuation factors are 1.30 ± 0.02 for PE versus 1.19 ± 0.02 for PC) indicating that magnetization transfer to PE occurs with higher efficiency. Signal intensity of the PS resonance was too low for a measurement of magnetization transfer.

**Photoillumination of ROS Disks**—The effect of photoactivation on interaction of DHA and saturated chains with rhodopsin was assessed by recording the $^1$H ST-MAS-NMR spectra of ROS disks as a function of time after photoactivation. Rhodopsin was completely bleached 30 min after the start of recordings by illuminating the spinning sample for 1 min with the light of a miniature light bulb inside the MAS stator. Sample heating from the infrared irradiation of the miniature light bulb was negligible as seen by the constancy of chemical shift of the water signal. Spectra were recorded continuously at 1.5-min intervals before ($A(t_0)$) and after photoillumination ($A(t)$), starting 2 min after exposing the sample to light. Fig. 4 reports the ratio of DHA attenuation $A(t)/A(t_0)$ recorded over 14 h.

Before photoactivation of rhodopsin, DHA attenuation was constant within small limits of experimental error. After bleaching, DHA attenuation $A(t)/A(t_0)$ increased within minutes to a value of 1.12 and decayed slowly over several hours back to the value of dark-adapted, unbleached ROS disks. The DHA data could be fitted to Equation 1,
where $A_{\text{max}}$ is the amplitude of attenuation increase, $\tau_1$ the time constant of formation of the state with increased attenuation, and $\tau_2$ the time constant of its disappearance. At a pH of 8.4 and a temperature of 30 °C, attenuation increased at $\tau_1 = 12 \pm 5$ min to a value of $\sim 12\%$ above the attenuation of dark-adapted rhodopsin and decayed at $\tau_2 = 350 \pm 50$ min to an attenuation similar to ROS disks before bleaching (Fig. 4A). The experiment was repeated twice on freshly prepared samples with similar results. Efficiency of rhodopsin bleaching in the rotor was confirmed by comparison with experiments on samples that were bleached for 1 min in the beam of a high-intensity green laser pointer before insertion into the MAS NMR probe. After temperature equilibration those samples gave identical results within the limits of experimental error.

When the temperature was lowered to 20 °C, the time constant $\tau_1$ increased to $45 \pm 5$ min and $\tau_2$ increased to $450 \pm 100$ min (Fig. 4B). At pH 6.4 and a temperature of 30 °C, DHA attenuation did not change with bleaching (Fig. 4C).

Signal attenuation of the resonance from mostly saturated chains at 1.3 ppm increased only by 2.5% after photoactivation, which is barely outside experimental error limits (data not shown).

A control experiment on 18:0-22:6n3-PC membranes without rhodopsin, conducted at otherwise identical conditions did not yield any time dependence of DHA signal intensity, confirming that the increased attenuation of the DHA resonance results from photoactivation of rhodopsin and not from drifts of signal amplification or temperature.

Upon adsorption of a photon, within a few milliseconds a temperature and pH-dependent equilibrium between Meta-I and Meta-II is established. Here, the increase of DHA attenuation after bleaching of rhodopsin had a rate of minutes. This indicates that dark-adapted rhodopsin, Meta-I and Meta-II rhodopsin intermediates experience similar interaction with DHA acyl chains. The changes must originate either from Meta-III or opsin. The latter was eliminated by following DHA attenuation on the mol fraction of polyunsaturated species was the same for 16:0d35-18:1n9-PC with 18:0d35-22:6n3-PC, 16:0d35-18:1n9-PC/18:0d35-22:6n3-PC, or 18:0d35-22:6n3-PS were conducted. Perdeuteration of the saturated palmitic and stearic acid chains eliminated their proton signals. As a consequence, the resonance at 1.3 ppm is only from the monounsaturated oleic acid (OA, 18:1n9), while the resonance at 2.8 ppm is from DHA only. Deuteration also suppressed intermolecular magnetization transfer between lipids. Independent of the molar ratio of poly- to monounsaturated lipids in the lipid matrix, 50% of hydrocarbon chains were always deuterated. Therefore at a random distribution of protonated and deuterated hydrocarbon chains, only one-fourth of all chain-chain interactions involved a pair of protonated chains, lowering the rates of lateral magnetization transfer within the lipid matrix by a factor of four. But even without deuteration, rates of lateral transfer of magnetization by cross-relaxation in the liquid crystalline state of 16:0:18:1-PC at ambient temperature (38) are several orders of magnitude lower than rates of lipid lateral diffusion (39). One is then able to study specificity of lipid-rhodopsin interaction without much concern about scrambling of results from magnetization transfer between lipids. As a result, the rates of magnetization transfer from rhodopsin to DHA or OA chains are well resolved.

In experiments conducted as a function of the mol fraction of polyunsaturated lipids, the attenuation of the DHA resonance increased rapidly between 0–25 mol % of polyunsaturated lipids in the mixture and reached saturation near a concentration of 50 mol % (Fig. 5). The dependence of DHA resonance attenuation on the mol fraction of polyunsaturated species was the same for 16:0d35-18:1n9-PC/18:0d35-22:6n3-PC, 16:0d35-18:1n9-PC/18:0d35-22:6n3-PC, and 16:0d35-18:1n9-PC/18:0d35-22:6n3-PS mixtures. However, the magnitude of DHA resonance attenuation varied from 1.25 ± 0.01 for 18:0d35-22:6n3-PC, to 1.51 ± 0.01 for 18:0d35-22:6n3-PE, and to 1.41 ± 0.01 for 18:0d35-22:6n3-PS. In contrast, OA attenuation remained constant at 1.3 up to a concentration of 75% polyunsaturated lipid in the mixture and decreased somewhat at higher DHA (lower OA) content.

The experimental data in Fig. 5 point at specific interaction of poly- and monounsaturated lipids with sites on rhodopsin. The concentration dependence has the appearance of ligand binding curves with a rapid onset of occupation of binding site(s) at low concentrations of polyunsaturated lipids. Affinity of the rhodopsin site for interaction with polyunsaturated DHA is such that 90% of sites are occupied at 50 mol % polyunsaturated lipid in the mixture. If sites would have no specificity for polyunsaturated lipid, a linear dependence, shown as a dashed line in Fig. 5B, is expected.

Furthermore, if poly- and monounsaturated lipids would have received magnetization from the same site(s) on rhodopsin, then the increase of DHA attenuation should have been accompanied by a decrease of OA attenuation as shown by a
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Dependence of DHA and OA signal attenuation as a function of the mol fraction of lipids with a DHA hydrocarbon chain. Membranes contained 250 lipids per rhodopsin. Rhodopsin was presaturated at 8.5 ppm, at a Bx field strength of 1 kHz, and a presaturation time of 500 ms. A, 16:0d31-18:1n9/18:0d35-22:6n3-PC; B, 16:0d31-18:1n9/18:0d35-22:6n3-PE; C, 16:0d31-18:1n9/18:0d35-22:6n3-PS; D, OA signal attenuation in samples: A, open squares; B, open triangles; and C, open diamonds. The solid lines are a fit to the concentration dependence according to Equations 2 and 3 with $K_{\text{DHA}} = 15 O_{\text{DHA}} = 12$, and $K_{\text{OA}} = 82$ and $O_{\text{OA}} = 8$, respectively. The dashed line in B shows a hypothetical concentration dependence of attenuation for sites on rhodopsin without specificity for DHA. The dashed line in D is an expected concentration dependence of OA resonance attenuation if DHA and OA had received magnetization from the same sites.

**dashed line** in Fig. 5D. Instead, OA attenuation remained constant and decreased at high concentrations of polyunsaturated lipid only. This requires that both polyunsaturated lipids (DHA) and monounsaturated lipids (OA) receive their magnetization from different sites on rhodopsin.

DHA signal attenuation was successfully fitted to a model of DHA and OA lipid binding. It was assumed that each rhodopsin molecule has a number of independent sites for binding of poly (DHA)- and monounsaturated (OA) lipids. Affinity of sites is described by the Law of Mass Action according to Equations 2 and 3,

$$K_{\text{DHA}} = \frac{O_{\text{DHA}} x_{\text{DHA}}}{x_{\text{DHA}} - x_{\text{DHA}} - x_{\text{DHA}}}$$

(Eq. 2)

$$K_{\text{OA}} = \frac{O_{\text{OA}} x_{\text{OA}}}{x_{\text{OA}} - x_{\text{OA}} - x_{\text{OA}}}$$

(Eq. 3)

where $x_{\text{DHA}}$ and $x_{\text{OA}}$ are the mol fraction of DHA and OA lipids in the mixture, respectively. The symbols $O_{\text{DHA}}$ and $O_{\text{OA}}$ represent the number of independent binding sites for DHA and OA lipids on rhodopsin, and $K_{\text{DHA}}$ and $K_{\text{OA}}$ are the corresponding association constants. The equations were solved for $x_{\text{DHA}} = x_{\text{DHA}} (O_{\text{DHA}}, K_{\text{DHA}})$ and $x_{\text{OA}} = x_{\text{OA}} (O_{\text{OA}}, K_{\text{OA}})$, and the variables $O_{\text{DHA}}, K_{\text{DHA}}, O_{\text{OA}}$, and $K_{\text{OA}}$ determined by fitting the solution to the experimental data. It was assumed that signal attenuation increases proportionally to the concentrations of $x_{\text{DHA}}$ and $x_{\text{OA}}$, respectively. The quality of the fit was judged by an error function defined as the sum of absolute values of linear deviations between experimental data points and the fit. Deviations at $x_{\text{DHA}} > 3$ were given a 5-fold higher weight to account for the importance of data points at low molar concentrations of DHA and OA lipids, respectively. A program for Mathcad (Mathsoft, Cambridge, MA) was written that probed the quality of fit for all possible combinations of variables. Results are summarized in Fig. 6 (see figure legend for details).

To address the concern that differences in signal attenuation of DHA or OA as a function of concentration could have resulted from changes in spin-lattice relaxation times $T_1$ that may occur with a change of lipid composition, we measured $T_1$ on selected samples and calculated the influence of relaxation on signal attenuation. The available lipid magnetization as a function of presaturation time was calculated by the recursive formula in Equation 4,

$$M_t = (M_{t-1} + (M_0 - M_{t-1}) \cdot \frac{1}{T_1} \exp \left( - \frac{t}{T_1} \right)) \cdot (1/\text{ms} - f)$$

(Eq. 4)

where $M_0$ is the total DHA or OA magnetization, $M_t$ the magnetization at presaturation time $t$, and $f$ the fraction of saturated magnetization per millisecond. Signal attenuation, probed by the π/2 hard pulse at the end of presaturation is then $A_t = M_t / M_0$. The measured linear dependence of signal attenuation as a function of presaturation time (Fig. 2C) could be well reproduced with the measured $T_1$ values in the range of 0.65–0.95 s and values of $f$ on the order of $10^{-3}$-ms. Remarkably, attenuation was rather insensitive to moderate changes of $T_1$. Deviations did not exceed the limits of experimental error of $T_1/8 = \pm 0.02$. Whereas signals with a larger $T_1$ saturate to a greater extent, those signals do not recover as fast during presaturation. The net result is that at low rates of presaturation, signal attenuation changes very little at modest changes of spin-lattice relaxation times.
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**DISCUSSION**

Previous studies have shown the importance of lipids with docosahexaenoic acid hydrocarbon chains and PE headgroups for rhodopsin function. In this article, the lipid-rhodopsin interaction with resolution for individual lipid species was investigated by ST-MAS-NMR. Highest rates of magnetization transfer were observed to PE lipids with DHA hydrocarbon chains. All rhodopsin photointermediates transferred magnetization preferentially to DHA-containing lipids, but Meta-III rhodopsin attenuated them the most. The specificity of lipid association was studied on rhodopsin in binary lipid mixtures of DHA- and OA-containing lipids. It was shown that polyunsaturated DHA and monounsaturated OA hydrocarbon chains associate specifically with a few, spatially distinct sites on rhodopsin. The PE headgroup increases rates of magnetization transfer to DHA chains but did not further raise specificity of lipid association. The data support a rhodopsin model with a heterogeneous lipid-rhodopsin interface, and lipids that interact specifically with certain sites on the protein surface.

**Saturation Transfer NMR (ST-MAS-NMR)**—In the ST-NMR experiment, $^1$H-$^1$H dipolar interactions drive the magnetization exchange at the lipid-protein interface. In other words, the rates of magnetization transfer are dependent on the distance between rhodopsin and lipid protons, the orientation to the magnetic field of the vector connecting those protons, and on motional correlation times of lipids in the protein-bound state. Therefore, acyl chains which interact more strongly with the protein or interact with a larger number of sites will receive more magnetization, and the corresponding resonances will be more attenuated.

A few limitations of the experiment must be pointed out as well. ST-NMR does not report the statistics of lipid composition in the first layer surrounding the protein. Results are biased toward protein sites from which magnetization transfer to lipids is particularly efficient. However, it is likely that lipids which interact more strongly with a limited number of protein sites are also present at an overall higher concentration. Furthermore, the experiment does not reveal directly the energetic significance of lipid-rhodopsin interactions. Off-rates of lipid release from the protein surface are of secondary importance as long as they are sufficiently high such that all lipids may reach the protein surface during the rather long presaturation time of 0.5 s. Indeed, experiments conducted as a function of temperature yielded higher rates of magnetization transfer with decreasing temperature. This indicates that rates of magnetization transfer by $^1$H-$^1$H dipolar interactions at the lipid-protein interface are the rate-limiting step, and not the off rate which decreases with decreasing temperature. A thermodynamic characterization of lipid-rhodopsin interaction requires conducting experiments as a function of lipid composition as reported in this article. Finally, the experiment does not allow identification of sites on the protein where lipids bind. The latter limitation may be overcome by isotopic labeling of the protein.

**Lipid Interaction with Dark-adapted Rhodopsin**—In the $^1$H ST-NMR experiment on ROS disks, a stronger attenuation of the DHA resonance compared with the resonance of mostly saturated chains was observed. This suggests stronger or more frequent interactions between DHA hydrocarbon chains and rhodopsin compared with interaction with stearic and palmitic acid chains.

Investigation of binary mixtures revealed that DHA and OA receive magnetization from distinct areas on rhodopsin reported here as poly- and monounsaturated sites (see Fig. 8). Each site is characterized by a high affinity for a particular lipid. For instance, at equal concentrations of poly and monounsaturated species, 90% of the polyunsaturated sites are occupied by DHA ($K_{DHA} = 21$). The monounsaturated sites had even higher specificity ($K_{OA} = 140$). But this higher specificity did not
translate into higher rates of magnetization transfer to OA chains. The preferential saturation of DHA chains, in particular when linked to a phospholipid with a PE headgroup, most likely indicates that DHA penetrates deeper into the rhodopsin molecule as suggested by molecular simulations (40). It was also observed that hydrocarbon chains with multiple double bonds separated by methylene groups do fit well to the backbone of transmembrane α-helices (40).

Higher rates of magnetization transfer to PE were seen in both the \(^{31}\)P ST-MAS-NMR experiments with \(^{1}H-^{31}\)P cross-polarization, and in the \(^{1}H\) ST-MAS-NMR experiments on DHA resonance attenuation in binary lipid mixtures. The experiments suggest that PEs interact tighter with rhodopsin and/or that PEs are enriched in the first lipid layer surrounding the protein. Previous ESR experiments did not report such a preference for interaction with a particular lipid species (41). However, the ESR study only investigated selectivity by lipid headgroups, not by composition of hydrocarbon chains. Therefore, the stronger attenuation of the PE phosphorus resonance may have resulted from a stronger interaction of DHA chains with rhodopsin in combination with the higher fractional content of polyunsaturated chains in PE. It was reported that ~80% of all fatty acids in PE are DHA versus ~60% in PC (42).

Interestingly, the change of lipid headgroups only altered rates of magnetization transfer, not the thermodynamic properties of DHA interaction expressed as an association constant and a number of binding sites. Therefore it can be concluded that the polar headgroups only fine tune interactions, e.g. by allowing a closer approach of DHA to binding sites on rhodopsin or by slightly increasing residence time. But the principle features of interaction are controlled by the DHA chain. Possible DHA-rhodopsin interaction sites were recently reported by Grossfield et al. (25) based on a molecular simulation.

**DHA Interaction with Photointermediates of Rhodopsin**—The magnetization transfer rate from rhodopsin photointermediates to DHA and saturated acyl chains was investigated on bovine ROS disks after photobleaching inside the spinning MAS rotor. The primary observation has been that rates of magnetization transfer to DHA chains are always highest, independent of the state of photoisomerization. Measurement of the DHA attenuation shows a clear dependence on the time after illumination. There was no sudden increase of DHA attenuation after bleaching. Instead a slow increase, consistent with reported rates of Meta-III formation, was observed. Furthermore, the effect was observed only at high pH values that favored Meta-I formation. Therefore, the results confirm recent reports by Vogel et al. (43) and Zimmermann et al. (44) that Meta-III is formed from Meta-I only (see Fig. 7). The higher rates of magnetization transfer to Meta-III indicate that rhodopsin helices in Meta-III are more accessible for interaction. The resonance of mostly saturated hydrocarbon chains had no such dependence of signal attenuation on photoactivation, indicating that only interaction with the polyunsaturated DHA is changing.

CONCLUSIONS

We have shown that magnetization transfer to polyunsaturated DHA chains is higher than to monounsaturated or saturated chains. Furthermore, lipid headgroups modify rates of magnetization transfer from rhodopsin to DHA with highest rates observed for PE. Moreover, ST-NMR experiment detected existence of distinct sites for interactions with polyunsaturated DHA or monounsaturated OA at those sites appears to be responsible for the specific magnetization transfer to DHA and OA from those sites. Differences of DHA attenuation as a function of lipid headgroups were observed as well. The DHA-specific sites have highest rates of magnetization transfer to PE (\(\rho_{PE}\)), followed by PS (\(\rho_{PS}\)), and PC (\(\rho_{PC}\)).
example, the endogenous ligands of the cannabinoid receptor, 2-arachidonylglycerol (2-AG) and arachidonylethanolamide (anandamide) are lipid-like substances with polyunsaturated arachidonyl chains (45, 46). The binding affinity of endogenous ligands may be orders of magnitude lower than affinity of synthetic ligands, but their concentration in tissue is higher as well, which compensates for lower affinity. It is conceivable that interaction with particular lipid species of even lower specificity play an important role in rhodopsin activation. Rhodopsin activation involves modification of interhelical contacts caused by a change of helix tilt angles, coupled helix rotations as well as a bending of helix segments. All of those structural changes have in common that they depend on the strength of helix-helix interactions. A penetration of DHA molecules between helical segments would displace helix-helix contacts and thereby facilitate the transition to the Gt-binding competent Meta-II state. The NMR data indicate that such specific associations between polyunsaturated lipids and rhodopsin are transient. Boundary lipids exchange with those away from the protein on a timescale of milliseconds or shorter. In the light of results presented in this article, the existence of such specific associations is indistinguishable. However, the location of interaction sites on rhodopsin as well as the energetic significance of those interactions for rhodopsin activation remain to be determined. We envision that the lipid matrix influences rhodopsin function both via continuum properties of the lipid matrix as well as via specific association of lipids with sites on rhodopsin.

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