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Selective Interface Detection: Mapping Binding Site Contacts in Membrane Proteins by NMR Spectroscopy

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Interactions with proteins are at the root of all living systems. In large molecules, such interactions usually involve only a small fraction of the total molecular surface area, for example, the ligand binding pocket of a large receptor protein. NMR methods for selective observation of intermolecular contact surfaces can simplify spectra while targeting a region of biological interest. Here, we propose a method for selective observation of interfacial 3−7 Å contacts between isotopically ^{13}C-labeled and unlabeled moieties by solid state magic angle spinning (MAS) NMR. We demonstrate this new technique in an application to an unlabeled membrane-embedded G-protein coupled receptor (GPCR), rhodopsin (Rho), containing the uniformly ^{13}C-labeled ligand, 11-*cis*-retinal.

G-protein coupled receptors are important signal transduction proteins, constituting a large family of genes with several subfamilies.[1] They are ubiquitous and diverse in multicellular organisms, mediating communication across membranes. They thus play central roles in a wide range of biological processes, including synaptic signaling, inflammation, hormone response, and vision.[1] Positioned at the cell surface, they are attractive targets for pharmaceutical intervention. Identification of the binding site ligand−receptor interactions is of particular interest for structure-based drug design.

GPCRs are integral membrane proteins with seven transmembrane α-helices. They are difficult to study due to a lack of overexpression systems, low solubility, and poor crystallizability. Embedded in a native lipid environment, they are too large for studies by solution state NMR methods. Rho is a GPCR with a covalently bound ligand, 11-*cis*-retinal. It is readily purified from cow eyes and has been studied extensively.[2] It is still the only GPCR to be crystallized.[3] Structural studies of other GPCRs have generally used NMR approaches, but they remain few and far between.[4] For Rho, structural studies involving selectively ^{13}C-labeled ligands have been used to gain atomic level details of the ligand structure in the binding site and to interpret the placement, size, and polarity of nearby charges.[5,6] Additional studies are revealing the light-activated signaling cascade.[7,8]

Here, we present ^{1}H−^{13}C HetCor spectra of Rho obtained with a new method: selective interface detection spectroscopy (SIDY). Synthesis of ^{13}C-labeled retinal and preparation of Rho have been described elsewhere.[5] The SIDY pulse sequence is shown in Figure 2 and described in detail in the Supporting Information. It begins with REDOR dephasing to remove all ^{1}H{^{13}C} signals from the HetCor t_1 dimension.[9] This effectively silences the $^{1}H_{lig}$. Dephasing is followed by a two-step magnetization transfer process in which the dephased ^{1}H{^{13}C} are used to detect nearby ^{1}H{^{12}C, N, O}, primarily on the protein. This consists of ^{1}H−^{1}H spin diffusion followed by a very short cross polarization (CP) mixing period for selective observation of ^{13}C{^{1}H}, particularly the $^{13}C_{lig}$. During spin diffusion, the ^{1}H{^{13}C} of the ligand are repolarized by the undephased ^{1}H{^{12}C, N, O} of the surrounding protein and lipids, which contain the t_1 chemical shift information. The spin diffusion and CP periods are kept short to minimize nonselective ^{1}H−^{1}H interactions. This two-step transfer mechanism minimizes relaxation effects and RF heating relative to earlier approaches,[9] as required for large, membrane-bound systems. Zero or double quantum ^{1}H−^{1}H mixing methods could also be used to obtain the $^{1}H_{GPCR}$−$^{1}H_{lig}$ contacts.[11]

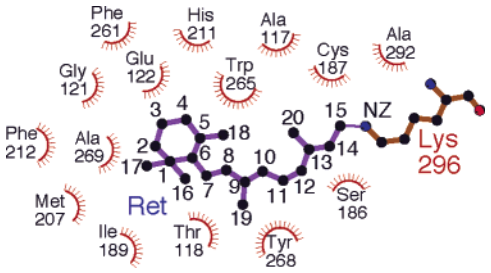

Figure 1. Retinal numbering scheme and closest amino acid contacts in the binding site. Contacts between molecule B of crystal structure 1F88 and retinal were derived with LPC software.[10]

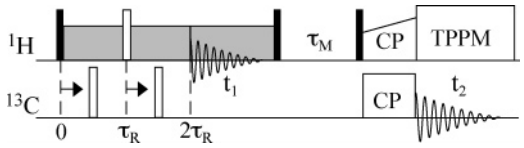

Figure 2. SIDY method for obtaining long distance correlations in HetCor spectra. First, REDOR dephasing of the ^{13}C-attached protons is achieved over $2\tau_R$ (→, typically 40 ms). The t_1 period is followed by active ^{1}H−^{1}H mixing (τ_M 200−800 μs). Finally, a short CP period (50−80 μs) yields the interfacial ^{1}H−^{13}C contacts. Filled and open rectangles are $\pi/2$ and π pulses, respectively. Gray boxes show periods of Lee−Goldberg (PMLG) decoupling executed with a 3.1 ms 90° pulse, which was optimized on adamantane.[12] TPPM decoupling was used in t_2.

We have compared the SIDY method with two other long distance HetCor methods in experimental applications to Rho. Without REDOR dephasing, a long (4 ms) LGCP contact time yields many ^{1}H−^{13}C correlations. These correspond primarily to $^{1}H_{lig}$−$^{13}C_{lig}$ contacts, although a few, weak $^{1}H_{GPCR}$−$^{13}C_{lig}$ contacts are detected. Similar results are obtained with a pulse sequence using ^{1}H−^{1}H spin diffusion and a short CP contact time (see Supporting Information). In general, correlations due to $^{1}H_{lig}$−$^{13}C_{lig}$ interactions dominate the ^{1}H spectrum, leaving little room to resolve the desired $^{1}H_{GPCR}$−$^{13}C_{lig}$ signals.

A ^{1}H−^{13}C HetCor spectrum obtained with SIDY is shown in Figure 3. A 1D CPMAS spectrum with assignment is shown above Figure 3. Large peaks due to lipid natural abundance ^{13}C lead to some spectral overlap in both the 1D and SIDY spectra. The resolved $^{13}C_{lig}$ correlations are solely due to $^{1}H_{GPCR}$−$^{13}C_{lig}$ interac-

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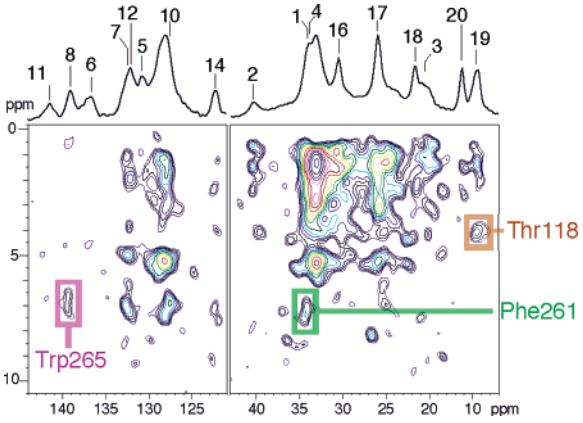

Figure 3. SIDY spectrum obtained from Rho at 223 K on a 750 MHz WB Avance spectrometer (Bruker, Karlsruhe, Germany) with 400 μs ^{1}H−^{1}H spin diffusion and 50 μs CP contact time. The spin rate was 11 223 Hz; 200 transients were combined for each t_1 slice. The t_2 data were truncated to 800 points, zero-filled to 2048, and windowed with 120 Hz of exponential broadening. The 192 points acquired in t_1 were linear predicted by 64 points using 40 coefficients. A 90° shifted sin^2 function was applied, and the data were zero-filled to 1024 points before Fourier transformation. A 1D CPMAS spectrum with the $^{13}C_{lig}$ assignment is shown above. Assignment and chemical shift referencing are based on ref 5.

tions and allow new insight into the binding site contacts in the native protein. In particular, many distinct $^1H_{GPCR}-^{13}C_{lig}$ correlations with the aliphatic carbons of the ligand ionone ring (1−6 and 16−18 in Figure 1) are resolved, indicating strong interactions with specific protein residues. This is consistent with studies of ligand binding and of the protein activation mechanism, which indicate that the ionone ring is important for selective binding and activation.[7,14]

The ligand C4 resonance shows a strong correlation at 7.3 ppm. There is overlap with a lipid resonance and the ligand C1 resonance in this ^{13}C spectral region. However, C1 is unprotonated and does not contribute to the SIDY spectrum. Also, the lipid ^{1}H spectrum has no signals in the ^{1}H chemical shift region near 7.3 ppm. Consequently, the observed correlation can be ascribed to C4 contacting an aromatic protein side chain, such as Phe261 or Trp265 (Figure 3). The SIDY data alone do not allow a sequence-specific assignment, but models and mutagenesis data can resolve the possibilities, even in the absence of further structural information.

$^1H_{GPCR}-^{13}C_{lig}$ correlations with the polyene carbons (7−15 and 19−20 in Figure 1) also reveal specific protein−ligand interactions. C19 shows strong correlations with aliphatic carbons and with either a backbone H-α or an H-bonding side chain at 4.1 ppm. The crystal structure shows a 3 Å contact with the Thr118 OH. C8 has a $^1H_{GPCR}-^{13}C_{lig}$ correlation with an aromatic residue. In Figure 3, we attribute this to Trp265 (see also Supporting Information).

In general, SIDY and other HetCor spectra reveal fewer correlations with the polyene chain than with the ionone ring. The rhodopsin X-ray crystal structure shows little difference between the distances of closest approach of the protein side chains to the ionone ring and polyene carbons (3.2−3.4 and 3.6−3.7 Å, respectively). The consistently low intensity of the $^1H_{GPCR}-^{13}C_{lig}$ polyene correlations may, therefore, be due to inefficient ^{1}H−^{1}H spin diffusion to this part of the ligand or to local disorder or motion in the protein.[15]

In particular, the Schiff base proton is anomalous. A strong signal is observed from this proton in HetCor spectra of crystalline retinylidene Schiff base model compounds (data not shown). In a series of SIDY and HetCor applications to Rho, however, this proton is not identified. Motion of the Schiff base ^{1}H on the NMR time scale could be quenching spin diffusion. This is consistent with studies of bacterio−rhodopsin, which have shown high ^{1}H mobility in this protein region. In Rho, the Schiff base ^{1}H is known to exchange, but the time scale has not been measured.[16] Our observations suggest dynamics on the millisecond time scale at −50 °C.

The $^1H_{GPCR}-^{13}C_{lig}$ contacts observed with SIDY are comparable to those obtained in experiments that rely on selective protein isotope labeling based on prior structural knowledge.[17] Here, we use unlabeled protein from a natural source. Furthermore, the SIDY method reveals many contacts in a single experiment. This method will be useful for mapping ligand binding sites in uncrystallizable membrane proteins, even when only small amounts of unlabeled protein are available. Specific ligand−protein contacts can be identified and possibly assigned based on additional data. This is primary, crucial information for structure-based drug design. The method can also be readily applied to other intermolecular contact surfaces, including protein−protein interfaces.

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Supporting Information Available: Additional pulse sequences and spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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