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Accurate Measurements of $^{13}C-^{13}C$ *J*-Couplings in the Rhodopsin Chromophore by Double-Quantum Solid-State NMR Spectroscopy

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Rhodopsin is a G-protein coupled receptor (GPCR) responsible for dim light vision in vertebrates. Rhodopsin consists of a seven-helix transmembrane protein bound by a protonated Schiff base (PSB) linkage to the 11-*Z*-retinylidene chromophore, which is the primary light receptor.[1] On photoactivation, the retinylidene chromophore is isomerized to the all-*E*-retinylidene configuration. This process is exceptionally specific and fast, and it is not fully understood how the protein environment stereoselectively steers and accelerates the photoisomerization process. NMR estimations of $^{13}C-^{13}C$ distances within the retinylidene chain suggested that the PSB positive charge is partially delocalized and stabilized near the isomerization site by polar protein side chains and an associated water molecule.[2] However, density functional calculations did not support this hypothesis.[3]

To obtain an independent estimation of the bond conjugation in the retinylidene chromophore, we have developed a double-quantum-filtered spin−echo (DQF-SE) NMR method to measure one-bond $^{13}C-^{13}C$ *J*-couplings for large biomolecules in the solid state. The $^1J_{CC}$ values are sensitive to the bond order, and double bonds display couplings around 15 Hz larger than single bonds. Accurate measurements of $^1J_{CC}$ couplings should therefore provide an unambiguous mapping of the bond conjugation within the retinylidene chain. We have applied this method to six different $^{13}C_2$-labeled rhodopsin isotopomers, namely [9,10-$^{13}C_2$], [10,11-$^{13}C_2$], [11,12-$^{13}C_2$], [12,13-$^{13}C_2$], [13,14-$^{13}C_2$], and [14,15-$^{13}C_2$]-retinylidene rhodopsin.

It has recently been shown that homonuclear *J*-couplings may be estimated accurately by spin−echo modulations in solids, even though the size of these couplings is often less than the line width.[4,5] To measure one-bond $^{13}C-^{13}C$ *J*-couplings in rhodopsin, we have combined the spin−echo technique with symmetry-based double-quantum dipolar recoupling[6] to suppress the natural abundance ^{13}C background, which otherwise obscures the signals of interest. This new NMR technique allows the measurement of *J*-couplings, and hence bond orders, with high accuracy in large, noncrystalline biomolecules.

The DQF-SE pulse sequence is shown in Figure 1 and consists of a preparation block, a DQ filtration block, and a spin−echo block. Magic-angle spinning (MAS) is used to improve the sensitivity and the resolution. The preparation module consists of ramped $^1H-^{13}C$ cross-polarization followed by a strong 90° pulse (90° out-of-phase with the spin-locking field) to enhance the longitudinal ^{13}C magnetization. The DQ filtration module consists of two intervals

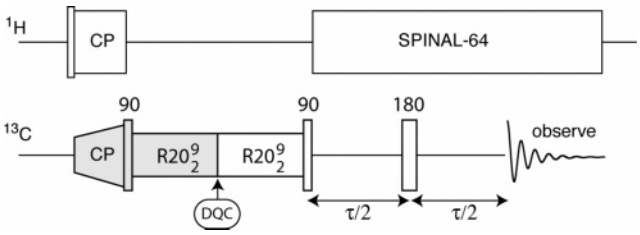

Figure 1. Double-quantum-filtered spin−echo pulse sequence for the measurement of *J*-couplings in MAS $^{13}C_2$-labeled biomolecules. The shaded pulse sequence elements are given a four-step phase cycle. Note the absence of an ^1H decoupling field during the $R20_2^9$ recoupling elements.

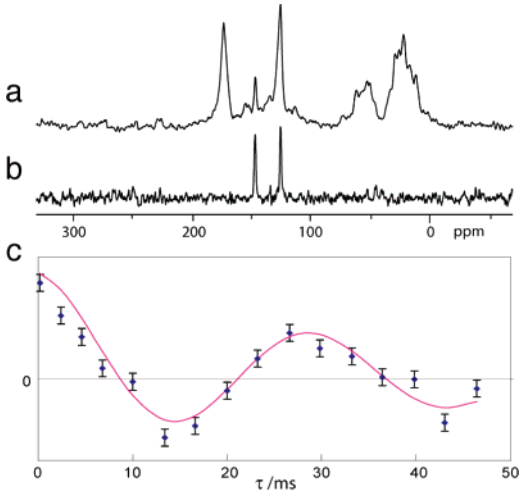

Figure 2. MAS ^{13}C NMR spectra of [9,10-$^{13}C_2$]-retinylidene rhodopsin, at a spinning frequency of 10.00 kHz, a field of 9.4 T, and a temperature of 170 K. (a) Cross-polarization spectrum. (b) Double-quantum-filtered spectrum showing the peaks from the ^{13}C labels. (c) Experimental double-quantum-filtered signal intensities as a function of the echo interval τ and a fitted echo modulation curve (solid line).

of symmetry-based $R20_2^9$ recoupling[6] to excite DQ ^{13}C coherences in the $^{13}C_2$-labeled species and to reconvert these coherences into observable magnetization. Four-step phase cycling is used to suppress signals from isolated ^{13}C nuclei that cannot support DQ coherence, thereby removing the natural abundance ^{13}C background signal, as shown in Figure 2b. The $R20_2^9$ sequence consists of a repeating pulse pattern $90_{81}270_{261}90_{-81}270_{-261}$ where the flip angles and phases are specified in degrees, and the nutation frequency under the rf field is 10 times the MAS frequency.[6] This sequence

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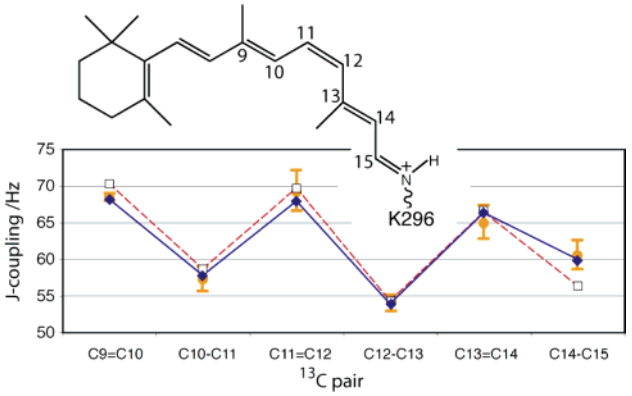

Figure 3. Filled circles with error bars: $^1J_{CC}$ values for the six rhodopsin isotopomers and their confidence limits. The inset shows the numbering scheme of the 11-*Z*-retinylidene chromophore. Diamonds and solid line: $^{13}C-^{13}C$ *J*-couplings for all-*E N*-*tert*-butyl retinylidene imine triflate in solution. Open squares and dashed line: $^{13}C-^{13}C$ *J*-couplings for all-*E* retinal in solution.

provides efficient DQ recoupling at a moderate MAS frequency without requiring a ^1H decoupling field.[7]

The DQ-filtered longitudinal magnetization is transformed into transverse magnetization by a second strong 90° pulse. A $\tau/2 - 180 - \tau/2$ spin−echo is performed in the presence of a ^1H decoupling field, and the ^{13}C NMR signal is detected. The rotor-synchronized spin−echo interval τ is varied in a series of experiments, and the oscillatory modulation of the ^{13}C signal is recorded. We used carefully adjusted SPINAL-64[8] proton decoupler modulation during the echo interval to prolong the echo decays.[9] An experimental spin−echo modulation curve for [9,10-$^{13}C_2$]-retinylidene rhodopsin is shown in Figure 2c.

Duma et al.[5] showed that the spin−echoes of isolated spin-pair systems in MAS solids obey the equation

$$s(\tau) = p \exp(-\tau/T_2^0) + (1 - p) \cos(\pi J \tau) \exp(-\tau/T_2^J)$$

The *J*-coupling is estimated by fitting this equation to the experimental data, adjusting the parameters T_2^0, T_2^J, p, and J. The confidence limits on J are derived by taking into account the experimental noise (see Supporting Information). The *J*-coupling estimates for the six rhodopsin isotopomers, and their confidence limits, are displayed graphically as a function of chain position in Figure 3. This figure also shows solution-state *J*-couplings measured for a PSB model compound (all-*E N*-*tert*-butyl retinylidene imine triflate[10]), and all-*E*-retinal, which has an aldehyde group instead of the protonated PSB moiety.[11] The *J*-couplings of the PSB model compound were measured by solution-state double-quantum NMR (see Supporting Information).

The measured *J*-couplings follow a zigzag pattern and correlate well with the alternation of single and double C−C bonds in the conjugated retinylidene chain. Some perturbations of the zigzag are clearly visible. At the end of the chain, the $^1J_{CC}$ value for the 14−15 single bond is enhanced in both rhodopsin and the PSB model compound, relative to retinal. This enhancement may be attributed to the positively charged PSB neighbor, which creates a conjugation defect. The penetration of the PSB positive charge into the end of the chain is also reflected in the chemical shift values[12] and the $^{13}C-^{13}C$ distances.[2]

The $^1J_{CC}$ values for rhodopsin and the PSB model compound agree in all positions within experimental error. The *J*-couplings therefore provide no indication that the protein environment significantly perturbs the electronic structure of the conjugated system, apart from the localized effects of the PSB at the chain terminus. These data therefore support the conclusions of Okada et al.[3] who argued, on the basis of X-ray diffraction and density functional calculations, that the bond alternation in the retinylidene chromophore is insensitive to the protein environment.

The fact that the dipole−dipole coupling[2] and chemical shift[12] measurements both indicate a disruption of conjugation penetrating deep into the conjugated chain, while a corresponding perturbation of the *J*-couplings is not observed, suggests that a single "bond order" parameter is too simplistic to describe the interplay of electronic structure, chemical shifts, internuclear distances, and *J*-couplings in a conjugated system. Quantum computations are planned to clarify this relationship.

In summary, we have developed and applied a solid-state NMR experiment that allows accurate and robust measurements of one-bond *J*-couplings in large noncrystalline macromolecules, such as membrane proteins. The magnitudes of *J*-couplings monitor very local features of the electronic bonding structure. In the case of rhodopsin, our measurements support the thesis that the protein environment does not perturb the chromophore electronic structure significantly in the vicinity of the isomerization site.

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Supporting Information Available: Numerical values of the *J*-couplings, full citation for ref 2, details of retinal synthesis and sample preparation, choice of NMR methodology, experimental NMR parameters, pulse sequence code, solid-state and solution-state NMR spectra, spin−echo modulation curves, and confidence limit analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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