INEPT-Based Separated-Local-Field NMR Spectroscopy: An Unique Approach to Elucidate Side Chain Dynamics of Membrane-Associated Proteins

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List of abbreviations used in the main text is given below.

BLEW: Burum, Linder, Ernst Windowless

Cyt-b_5: Cytochrome b_5

DHPC: 1, 2-dihexanoyl-*sn*-glycero-3-phosphatidylcholine

DMPC: 1, 2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine

NMR: Nuclear Magnetic Resonance

MAS: Magic Angle Spinning

PELF: Proton Evolved Local Field

RampCP: Ramped Cross-Polarization

RDCs: Residual Dipolar Couplings

RINEPT: Refocused Insensitive Nuclei Enhanced by Polarization Transfer

RF: Radio Frequency

SLF: Separated Local Field

SPINAL: Small Phase Incremental Alternation

WIM: Windowless Isotropic Mixing

Preparation of magnetically-aligned bicelles

Details on the preparation and characterization of magnetically-aligned bicelles containing cytochrome b5 can be found in our earlier publication.[1] In this study, bicelles with a q = [DMPC]/[DHPC] value of 3.5 were used. The desired mixture of lipid and detergent was hydrated with an appropriate amount of HEPES buffer (10 mM HEPES, pH = 7.0) to obtain the desired wt% of lipids, typically ~ 50 wt%, for the reconstitution of a mutant-cytrochrome b_5. The hydrated lipid-detergent mixture was gently vortexed after every freeze/thaw cycle until an optically clear solution was obtained. The procedure to reconstitute a mutant-cytochrome-b5 is given below.

Reconstitution of mutant cytrochrome b_5 in bicelles

Uniformly ^{15}N-labeled mutant-cytochrome b_5 was expressed and purified according to previous protocols.[2] The protein was concentrated to a stock solution of 4.3 mM. The amount of protein used was calculated according to the desired lipid to protein ratio. A 300:1 DMPC:protein ratio was used in this study. The protein was reconstituted into bicelles by adding an appropriate amount of the protein stock solution to bicelle solution and the final lipid concentration was diluted to 25 wt% and the final volume was 160 μL. The solution was gently vortexed to ensure proper mixing of protein and lipids and subsequently transferred to a glass tube of 4 cm in length. The final sample consisted of ~40 mg of bicelles and 3 mg of a mutant cytochrome b_5. The tube was closed firmly with Teflon tape to avoid any leakage or dehydration inside the probe. The sample was then equilibrated for about 30 minutes inside the magnet at the 35°C prior to signal acquisition. NMR experimental details are given below.

NMR experiments

NMR experiments were carried out on a Varian Infinity-600 MHz solid-state NMR spectrometer using a 4 mm triple-resonance magic-angle spinning (MAS) probe under static sample conditions. The ^{1}H and ^{15}N resonance frequencies were 599.8 and 60.78 MHz respectively. RF pulse sequences and phase cycling used in this study are given in Figure S1. Typical parameters used to obtain 2D DREPT spectra of magnetically-aligned bicelles containing a uniformly ^{15}N-labeled mutant-cytochrome-b5 are: 5 μs 90° pulse, 40 kHz ^{15}N spectral width, 28 t_1 increments, 2000 scans and a 3s recycle delay. 1D and 2D spectra are presented in the main text (Figures 1 and 2 respectively). The complete amino acid sequences of the wild-type and the mutant-cytochrome-b5 are given in Figure S2. Typical parameters used to obtain 2D HETCOR spectra of the above-mentioned sample (Figure S3) are: 5 μs 90° pulse, 40 kHz ^{15}N spectral width, 20 t_1 increments, 800 scans and a 3s recycle delay. For both HETCOR and DREPT experiments, a 35 kHz RF field strength was used for both BLEW-12[3] and SPINAL-64[4] decoupling. All spectra were processed using NMRpipe[5] and analyzed using SPARKY[6].

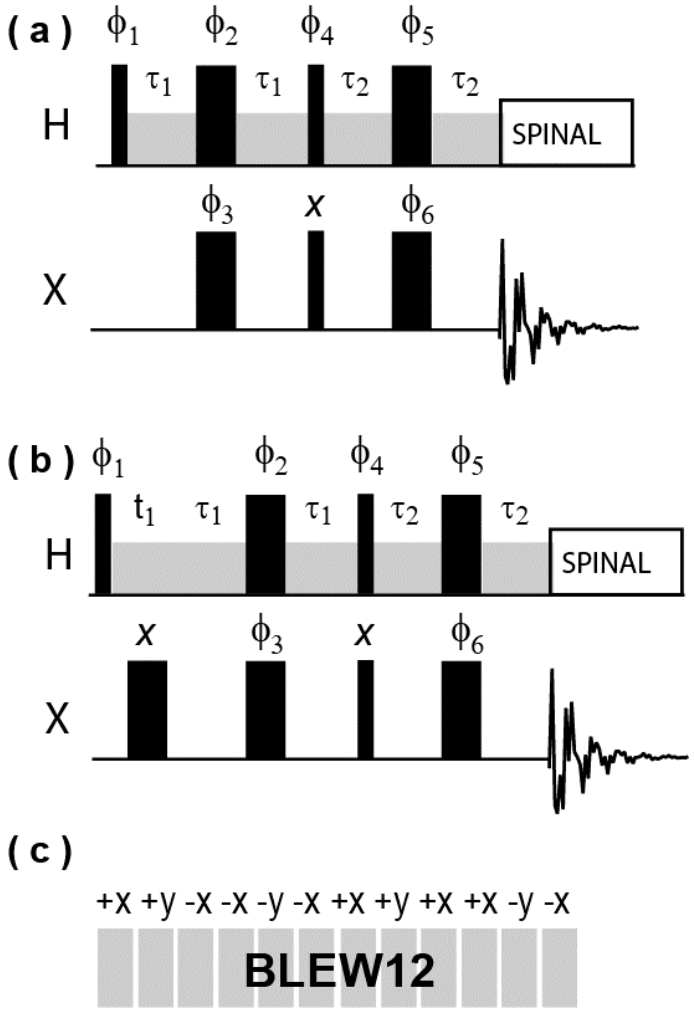

Figure S1. Schematics of RF pulse sequences. (a) A 1D DREPT pulse sequence used in this study. The DREPT pulse sequence is based on the commonly used refocused-INEPT (or RINEPT) sequence.[7] A proton homonuclear decoupling sequence (BLEW-12 sequence indicated in grey rectangles) is inserted in the delays to suppress proton-proton interactions; BLEW-12 is repeated without any delay in the τ_1, τ_2 and in the incrementable t_1 periods. The decoupling sequence, BLEW-12, is a windowless multiple pulse sequence consisting of twelve 90^0 pulses (with phases x, y, -x, -x, -y, -x, x, y, x, x, -y, -x). By decoupling ^{1}H-^{1}H dipole-dipole interactions, the spin systems in an aligned static solid can be made to behave similar to an isotropic system since each *IS* spin system experiences a single heteronuclear dipolar coupling value, which depends on the orientation of the *I-S* vector with respect to the external magnetic field of the spectrometer. A full theoretical description of the pulse sequence is given below. Phases of RF pulses in the sequences are as follows: ϕ_1=y, y, y, y, -y, -y, -y, -y; ϕ_2=ϕ_3=ϕ_5=ϕ_6=x, -x; ϕ_4=x, x, -x, -x. A multiple of 8 scans needs to be co-added to complete the RF phase cycle with the alternation of the receiver phase after every four scans. (b) A 2D HETCOR pulse sequence based on the DREPT pulse sequence was designed to observe the correlation of chemical shifts of *I* and *S* nuclei in aligned static samples. (c) The BLEW-12 homonuclear proton decoupling pulse sequence consisting of a series of twelve 90° pulses with the indicated RF phase.

DKDVKYYTLEEIKKHNHSKSTWLIKHHKVYDLTKFLEEHPGGEEVLREQAGGDATENFE

DVGHSTDARELSKTFIIGELHPDDRSKLSKPMETLITTVDSNSSWWTNWVIPAISALIVAL

MYRLYMADD

Figure S2. The amino acid sequence of the full-length wild-type rabbit cytochrome b_5. Secondary structures such as α-helix (blue) and β-sheet (green) are indicated. 8 amino acids that were deleted from the linker region of the wild-type cytochrome b_5 to obtain the mutant protein are highlighted in red. Since this protein reconstitutes readily in bicelles, it was chosen as a model system to demonstrate the efficiency of the 2D DREPT pulse sequence. The residues with their side chain identified in the DREPT spectrum are shown in yellow.

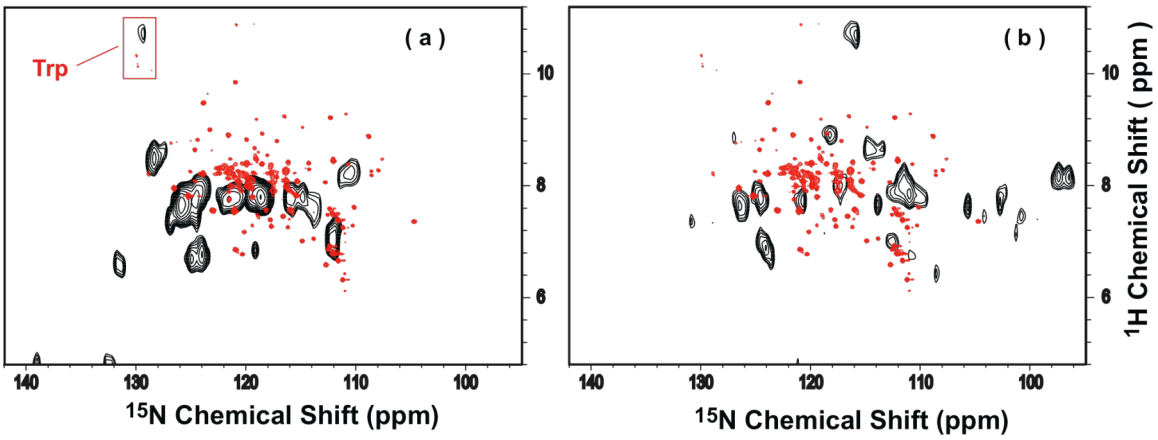

Figure S3. Assignment of ^{15}N resonances from amino acid side chains based on 2D $^{1}H/^{15}N$ HETCOR and 2D $^{1}H/^{15}N$ HSQC spectra. 2D DREPT-based $^{1}H/^{15}N$ HETCOR spectra (black) of a uniformly ^{15}N-labeled mutant-cytochrome b_5 embedded in DHPC:DMPC=3.5 bicelles were obtained using the pulse sequence given in Figure S1(b). These HETCOR spectra were recorded by setting the second delay period, τ_2, equal to 1 ms (a) or 80 μs (b), with 20 t_1 increments, 3s recycle delay and 800 scans per t_1 increment. The 2D DREPT-based $^{1}H/^{15}N$ HETCOR spectra (black) are compared with the 2D $^{1}H/^{15}N$ HSQC spectrum of the same protein embedded in 300mM DHPC micelles (red color). The HSQC spectra were recorded on a Bruker 600 NMR spectrometer at 35°C using 256 t_1 increments, 5s recycle delay and 32 scans per t_1 increment. Due to the high degree of order in an aligned bicelles sample, only resonances from the mobile region of the proteins were observed. In these cases, side chains of Trp and His were observed and identified due to their distinct proton chemical shift values.

S_{SC} order parameter profile

S_{SC} order parameter profiles of Arg and Trp side chains were simulated based on the "diffusion in a cone and orientational fluctuation" model as explained in the main text. Simulated results are given in Figure S4.

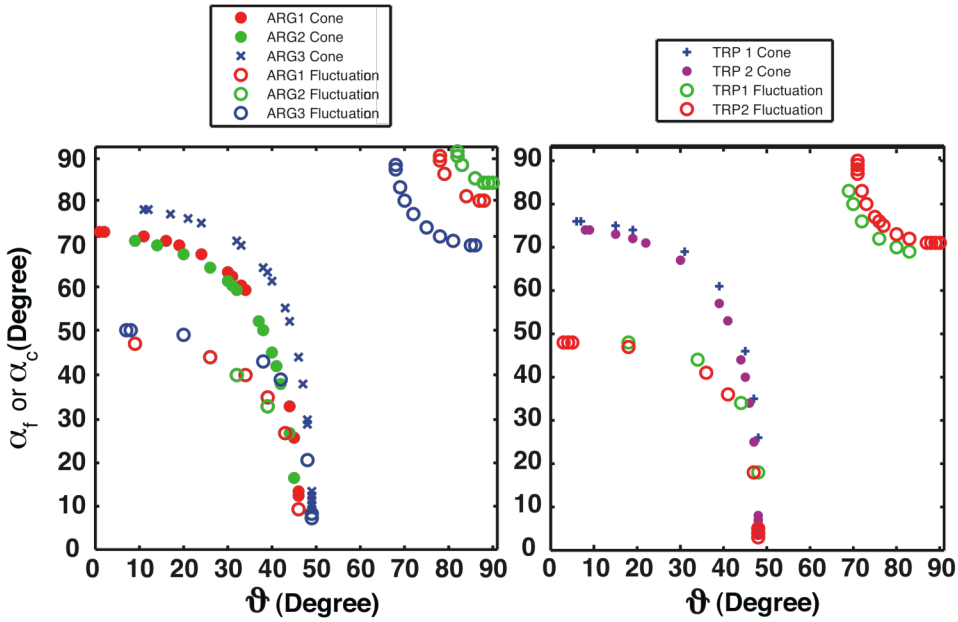

Figure S4. A plot showing all possible combination of angles between 0 to 90^0 that give rise to the observed value of the order parameter S_{SC} of Arg (left) and Trp (right) side chain. In this case, only angles from 0 to 90^0 were considered due to the symmetry of the system. For the case with α_f or α_c is equal to zero, it represents a rigid limit in which no fluctuation or wobbling motion is present in the side chain. In the rigid limit, the orientation of the Arg side chain is calculated to be 47° and the Trp side chain is calculated to be 50°. Numerical simulations were done using an in house MATLAB (version R2009b) script running on a dual core Macbook.

Theory of the DREPT pulse sequence

BLEW-12 multiple pulse sequence was used in the DREPT method as explained in Figure S1. The zeroth-order average Hamiltonian for the BLEW-12 homonuclear proton decoupling pulse sequence is given below in equation [S1] and the direction of its effective field is depicted in Figure S5.

$$H_{CS}^{0} = \frac{2}{3\pi}(2I_x + I_z)\Omega_I \qquad [S1]$$

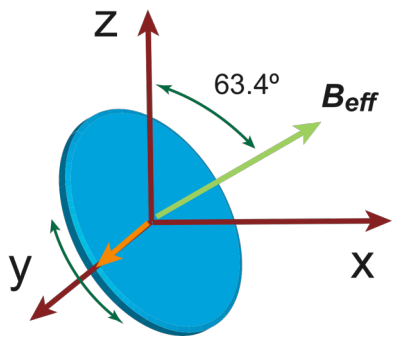

Figure S5. An illustration of the effective RF field under BLEW-12 decoupling. The magnetization in the plane (blue color) evolves under the effective RF field of BLEW-12.

While the proton homonuclear dipolar coupling averages to zero based on the zeroth-order approximation under BLEW-12, the heteronuclear dipolar coupling is scaled down according to the Hamiltonian given below in equation [S2].

$$H_{IS}^{0} = \frac{2}{3\pi}\omega_{IS}(4I_xS_z + 2I_zS_z) \qquad [S2]$$

I_i and S_i (where i=x, y or z) are components of angular momentum operators of I (typically protons) and S (usually low-gamma nuclei like ^{15}N or ^{13}C) spin nuclei respectively. The scaling factor, s, is calculated to be 0.475. The effective RF field is along an axis tilted by 63.4° from the z-axis in the x-z plane as shown in Figure S5. After a 90° pulse along the x direction, the proton magnetization lies along the y axis, as indicated by the orange arrow. The transversal proton magnetization evolves under the scaled heteronuclear dipolar coupling and chemical shift interaction in a plane perpendicular to the effective RF field as shown in Figure S5.

Density matrix analysis of the DREPT pulse sequence

In an aligned sample, each *IS* spin system experiences a single heteronuclear dipolar coupling value, which is based on the orientation of the IS internuclear vector with respect to the external magnetic field. Therefore, the density matrix of the DREPT pulse sequence is similar to the standard RINEPT pulse sequence, except that both chemical shift and heteronuclear dipolar coupling are scaled due the BLEW-12 homonuclear proton decoupling sequence. For a *IS* system, the density operator during the first delay period of the of DREPT pulse sequence is given below in equation [S3].

$$\sigma_1 = I_y \cos(2\pi s(J+D)\tau_1) + 2I_x S_z \sin(2\pi s(J+D)\tau_1) - S_z \quad \text{[S3]}$$

In this operator, the transverse proton magnetzation evolves under the sum of the heteronuclear scalar and dipolar couplings. In the PELF sequence, only the first

term I_y in equation [S3] is transferred to the directly bonded *S*-spin for detection by a cross-polarization or windowless isotropic mixing (WIM) sequence. On the other hand, in the DREPT pulse sequence, the application of two simultaneous 90° pulses on *I* and *S* RF channels converts the antiphase term I_xS_z in equation [S3] to an antiphase rare nuclear (^{13}C or ^{15}N) spin magnetization for detection. The corresponding density operator after this transfer of magnetization from protons to ^{15}N nuclei is given below in equation [S4].

$$\sigma_2 = 2I_zS_y \sin(2\pi s(J+D)\tau_1) + S_y \qquad \text{[S4]}$$

The S_y term is cancelled out by the phase cycling (see Figure S1 caption) and only the antiphase term I_zS_y term is detected after converting it into an inphase term by evolving under both heteronuclear scalar and dipolar couplings as given in the density operator below (equation [S5]).

$$\sigma_f = 2I_zS_y \sin(2\pi s(J+D)\tau_1)\cos(2\pi s(J+D)\tau_2) - S_x \sin(2\pi s(J+D)\tau_1)\sin(2\pi s(J+D)\tau_2) \qquad \text{[S5]}$$

Based on the final density matrix given above, the time-dependence of the observed *S*-spin signal intensity is given by the following equation [S6].

$$e^{-\tau_1/T_2^I} \sin(2\pi s(J+D)\tau_1) e^{-\tau_2/T_2^S} \sin(2\pi s(J+D)\tau_2) \qquad \text{[S6]}$$

Where T^{I}_{2} is the proton spin-spin relaxation time during the first delay period, while T^{S}_{2} is the *S* spin-spin relaxation time during the second delay period of the DREPT pulse sequence.

References

(1) Soong, R., Smith, P. E. S., Xu, J., Yamamoto, K., Im, S. C., Waskell, L., Ramamoorthy, A. *J. Am. Chem. Soc.*, **2010**, *132*, 5779-5788.

(2) Clarke, T. A., Im, S. C., Bidwal, A., Waskell, L., *J. Bio. Chem.*, **2010**, 279, 36809- 36818.

(3) Burum, D., Linder, M., and Ernst, R. R. *J. Magn. Reson.* **1981**,44, 173– 188

(4) Fung, B. M., Ermolaev, Y., and Yu, Y. *J. Magn. Reson.* **1999**,138, 28– 35

(5) Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., Bax, A., *J. Biomol. NMR*. **1995,** 6, 277-293.

(6) Goddard, T. D., Kneller, D. G., *SPARKY 3*, University of California, San Francisco

(7) Morris, G. A., Freeman, R. *J. Am. Chem. Soc.* **1979**, *101*, 760-762.