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Efficient ^{15}N - ^{13}C Polarization Transfer by Third-Spin Assisted Pulsed Cross Polarization Magic-Angle-Spinning NMR for Protein Structure Determination

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Abstract

We introduce a pulsed third-spin assisted recoupling experiment that produces high-intensity long-range ^{15}N - ^{13}C cross peaks using low radiofrequency (rf) energy. This Proton-Enhanced Rotor-echo Short Pulse IRradiATION Cross Polarization ($^{\text{PERSPIRATION}}\text{CP}$) pulse sequence operates with the same principle as the Proton-Assisted Insensitive-Nuclei Cross Polarization ($^{\text{PAIN}}\text{CP}$) experiment, but uses only a fraction of the rf energy by replacing continuous-wave ^{13}C and ^{15}N irradiation with rotor-echo 90° pulses. Using formyl-MLF and $\beta 1$ immunoglobulin binding domain of protein G (GB1) as model proteins, we demonstrate experimentally how $^{\text{PERSPIRATION}}\text{CP}$ polarization transfer depends on the CP contact time, rf power, pulse flip angle, and ^{13}C carrier frequency, and compare the $^{\text{PERSPIRATION}}\text{CP}$ performance with the performances of $^{\text{PAIN}}\text{CP}$, $^{\text{RESPIRATION}}\text{CP}$, and $^{\text{SPECIFIC}}\text{CP}$ for measuring ^{15}N - ^{13}C cross peaks. $^{\text{PERSPIRATION}}\text{CP}$ achieves long-range ^{15}N - ^{13}C transfer and yields higher cross peak intensities than the other techniques. Numerical simulations reproduce the experimental trends and moreover indicate that $^{\text{PERSPIRATION}}\text{CP}$ relies on ^{15}N - ^1H and ^{13}C - ^1H dipolar couplings rather than ^{15}N - ^{13}C dipolar coupling for polarization transfer. Therefore, $^{\text{PERSPIRATION}}\text{CP}$ is an rf-efficient and higher-sensitivity alternative to $^{\text{PAIN}}\text{CP}$ for measuring long-range ^{15}N - ^{13}C correlations, which are essential for protein resonance assignment and structure determination. Using cross peaks from two $^{\text{PERSPIRATION}}\text{CP}$ ^{15}N - ^{13}C correlation spectra as the sole distance restraints, supplemented with (ϕ, ψ) torsion angles obtained from chemical shifts, we calculated the GB1 structure and obtained a backbone RMSD of 2.0 Å from the high-resolution structure of the protein. Therefore, this rf-efficient $^{\text{PERSPIRATION}}\text{CP}$ method is useful for obtaining many long-range distance restraints for protein structure determination.

Graphical Abstract

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Supporting Information

Table of the atomic coordinates and chemical shift parameters used in numerical simulations;

Additional simulations of $^{\text{PERSPIRATION}}\text{CP}$ buildup for different flip angles;

Simulations of $^{\text{PAIN}}\text{CP}$ dependence on ^{13}C rf carrier frequencies;

Simulations of $^{\text{PERSPIRATION}}\text{CP}$ dependence on rotor-echo pulse field strengths;

SpinEvolution script for simulating $^{\text{PERSPIRATION}}\text{CP}$ buildup curves;

$^{\text{PERSPIRATION}}\text{CP}$ Bruker pulse program with phase cycles

Introduction

Magic-angle-spinning (MAS) solid-state nuclear magnetic resonance (SSNMR) spectroscopy has become instrumental for determining the molecular structures and characterizing the functions of many biological macromolecules, including membrane proteins¹⁻⁹, amyloid fibrils¹⁰⁻¹⁵, and cell walls of plants, bacteria and fungi¹⁶⁻²³. To resolve the signals of ^{13}C and ^{15}N -enriched proteins and carbohydrates, multidimensional correlation experiments are crucial. The majority of these correlation techniques achieve polarization transfer using distance-dependent homonuclear and heteronuclear dipolar couplings²⁴, so that cross peak intensities reflect inter-atomic distances in a semi-quantitative fashion. Since MAS averages dipolar couplings, a plethora of pulse sequences has been developed to recouple dipolar couplings to measure these correlation spectra and extract inter-atomic distances.

For protein resonance assignment, it is essential to correlate the amide ^{15}N signal with ^{13}C chemical shifts from the same residue as well as from the preceding residue. Both one-bond N-C α and N-CO cross peaks and multi-bond N-CX correlations to sidechain carbons are required for assigning the chemical shifts. Once resonance assignment is obtained, ^{15}N - ^{13}C correlations between residues that are well separated in the primary sequence are important for constraining the three-dimensional structure of the protein. Because of the low gyromagnetic ratios of ^{15}N and ^{13}C and the resulting weak dipolar couplings, multi-bond and long-range ^{15}N - ^{13}C cross peaks are difficult to detect with high sensitivity. Cross polarization (CP)²⁵ has been the most common approach for measuring ^{15}N - ^{13}C cross peaks, although pulsed methods based on Rotational-Echo Double-Resonance (REDOR) have also been used²⁶⁻²⁷. The double-CP (DCP)²⁸ and SPECIFICCP²⁹ experiments correlate ^{15}N and ^{13}C chemical shifts in a broadband and band-selective fashion, respectively, by simultaneous continuous-wave (CW) irradiation on the ^{13}C and ^{15}N channels while decoupling protons with high rf power. The ^{13}C and ^{15}N transverse rf field strengths satisfy the centerband Hartman-Hahn condition in DCP, while an effective-field sideband-matching condition is used in the SPECIFICCP technique to achieve selective transfer from ^{15}N to CA or ^{15}N to CO. Since both DCP and SPECIFICCP require multi-channel high-power rf irradiation for several milliseconds, these techniques put a significant demand on the NMR probe. Moreover, since both methods rely on direct ^{15}N - ^{13}C dipolar couplings for polarization transfer, ^{15}N cross peaks with sidechain carbons often require an additional ^{13}C - ^{13}C mixing period to detect. Although various homonuclear recoupling methods have

been used for ^{13}C mixing³⁰⁻³¹, ^{13}C spin diffusion remains to be the most common approach, and with increasing MAS frequencies, spin diffusion becomes inefficient, thus lowering the cross peak intensities.

Two alternative approaches have been developed in the last decade to measure ^{15}N - ^{13}C cross peaks with higher sensitivity and to longer distances. The proton-assisted insensitive nuclei cross polarization ($^{\text{PAIN}}\text{CP}$) approach³²⁻³³ relies on cross-terms between ^1H - ^{13}C and ^1H - ^{15}N dipolar couplings in the second-order average Hamiltonian to mediate polarization transfer between ^{15}N and ^{13}C . Because this technique relies on an intervening third spin, a proton, which has a much higher gyromagnetic ratio and hence stronger dipolar couplings, the ^{15}N -(^1H)- ^{13}C polarization transfer is more efficient than DCP and $^{\text{SPECIFIC}}\text{CP}$, which allows long-range cross peaks to be measured with higher sensitivity. During $^{\text{PAIN}}\text{CP}$ irradiation, proton-assisted ^{13}C - ^{13}C recoupling (PAR) also occurs³⁴⁻³⁶, which further increases the intensities of carbons that are far from the ^{15}N spin through relayed transfer. However, similar to DCP and $^{\text{SPECIFIC}}\text{CP}$, $^{\text{PAIN}}\text{CP}$ requires CW irradiation of all three nuclei. Although there are multiple matching conditions for $^{\text{PAIN}}\text{CP}$, the most commonly used and reliable condition involves rf field strengths of ~50 kHz for all three channels. These ^{13}C and ^{15}N rf powers are significantly stronger than typically used in the DCP and $^{\text{SPECIFIC}}\text{CP}$ experiments, which make $^{\text{PAIN}}\text{CP}$ even more rf intensive than the two predecessors.

The second approach that has been proposed for measuring ^{15}N - ^{13}C cross peaks is the Rotor-Echo Short Pulse IRradiATION Cross Polarization ($^{\text{RESPIRATION}}\text{CP}$)³⁷ experiment, which consists of short, rotor-synchronized pulses on the ^{13}C and ^{15}N channels that are interleaved with phase-alternating ^{13}C recoupling pulses. The first-order average Hamiltonian of $^{\text{RESPIRATION}}\text{CP}$ includes both first- and second-order Fourier components of the dipolar couplings, thus it achieves more efficient ^{15}N - ^{13}C polarization transfer than DCP and $^{\text{SPECIFIC}}\text{CP}$ ³⁸⁻³⁹. However, because $^{\text{RESPIRATION}}\text{CP}$ is a first-order recoupling sequence, long-distance ^{15}N - ^{13}C polarization transfer remains difficult, and most intensities are restricted to one-bond N-C α and N-CO correlations. Moreover, although no ^{15}N CW irradiation is used in $^{\text{RESPIRATION}}\text{CP}$, strong ^1H decoupling is required during the contact time, and the rf field strength of the phase-alternating ^{13}C recoupling pulses increases with MAS. Therefore, the rf power requirement of $^{\text{RESPIRATION}}\text{CP}$ remains high at fast MAS.

Drawing on the individual strengths of $^{\text{PAIN}}\text{CP}$ and $^{\text{RESPIRATION}}\text{CP}$, here we report a pulsed variant of the $^{\text{PAIN}}\text{CP}$ technique, which we call Proton-Enhanced Rotor-echo Short Pulse IRradiATION Cross Polarization ($^{\text{PERSPIRATION}}\text{CP}$). $^{\text{PERSPIRATION}}\text{CP}$ consists of the same moderate CW irradiation on the ^1H channel as in $^{\text{PAIN}}\text{CP}$, together with rotor-echo 90° pulses on the ^{13}C and ^{15}N channels as in $^{\text{RESPIRATION}}\text{CP}$, but no CW irradiation is applied on the ^{13}C or ^{15}N channel. Thus $^{\text{PERSPIRATION}}\text{CP}$ has a much lower rf duty cycle than $^{\text{RESPIRATION}}\text{CP}$ and $^{\text{PAIN}}\text{CP}$. We show by experiments and simulations that $^{\text{PERSPIRATION}}\text{CP}$ relies on cross-terms between ^1H - ^{13}C and ^1H - ^{15}N dipolar couplings and between ^1H - $^{13}\text{C}_1$ and ^1H - $^{13}\text{C}_2$ to achieve long-range ^{15}N - ^{13}C polarization transfer, as in $^{\text{PAIN}}\text{CP}$ and PAR. We compare the experimental ^{15}N - ^{13}C transfer characteristics of $^{\text{SPECIFIC}}\text{CP}$, $^{\text{RESPIRATION}}\text{CP}$, $^{\text{PAIN}}\text{CP}$, and $^{\text{PERSPIRATION}}\text{CP}$ using the model tripeptide formyl-MLF (f-MLF) and the microcrystalline $\beta 1$ immunoglobulin binding domain of protein G (GB1). We also

investigate the dependence of PERSPIRATION^{CP} on the ¹³C carrier frequency and pulse flip angle. Finally, we demonstrate the use of PERSPIRATION^{CP} for measuring 2D ¹⁵N-¹³C correlation spectra, and show that with ¹⁵N-¹³C cross peaks from two 2D spectra as the only distance restraints, we can already obtain a three-dimensional structure of GB1 with excellent agreement with the known high-resolution structure of this protein.

Materials and Methods

Sample Preparation

Uniformly ¹³C, ¹⁵N-labeled f-MLF was purchased from Cambridge Isotope Laboratories (Andover, MA) and center-packed into a 3.2 mm rotor without further purification. ¹³C, ¹⁵N-labeled GB1 was expressed in M9 minimal media containing uniformly ¹³C-labeled glucose and ¹⁵NH₄Cl according to previously published protocols ⁴⁰. The purified protein was dialyzed against pH 5.5 phosphate buffer to remove NaCl and reach the optimal pH for crystallization. The protein was then back-exchanged with D₂O and crystallized in a solution containing a 2:1 ratio of 2-methyl-2,4-pentanediol (MPD) to isopropanol (IPA) ⁴¹. Based on ¹H-¹⁵N CP spectra, we estimate that ~80% of labile protons are exchanged with ²H.

Solid-State NMR Experiments

All experiments were conducted on a 600 MHz (14.1 T) Bruker Avance III HD spectrometer using a ¹H/¹³C/¹⁵N 3.2 mm MAS probe. ¹³C chemical shifts were referenced to the 38.48 ppm CH₂ peak of adamantane on the TMS scale ⁴² while ¹⁵N chemical shifts were referenced to the 118.57 ppm peak of Leu in f-MLF on the liquid ammonia scale. Experiments on f-MLF and GB1 were conducted at set temperatures of 293 K and 278 K, respectively. The MAS frequency was 20 kHz for all experiments. At this MAS frequency, the actual sample temperature was estimated to be 10-20 K higher than the set point due to frictional heating ⁴³.

¹³C and ¹⁵N rf field strengths ranged from 10 to 50 kHz for the ¹⁵N-¹³C CP period (Table 1), while the ¹H irradiation field strengths were 38-63 kHz for the PAIN^{CP} and PERSPIRATION^{CP} experiments but 90-100 kHz for the SPECIFIC^{CP} and RESPIRATION^{CP} experiments. The ¹H CW irradiation field strengths were swept from 25 to 83 kHz when optimizing PAIN^{CP} and PERSPIRATION^{CP}; the field strengths that gave rise to the most uniform transfer to all ¹³C nuclei while maintaining high spectral sensitivity were chosen for the final experiments. During ¹³C detection, ¹H TPPM decoupling was applied with an rf field of 83 kHz ⁴⁴. 2D ¹⁵N-¹³C correlation spectra of f-MLF were measured using a 12 ms ¹³C acquisition time and a maximum ¹⁵N evolution time of 7 ms, while the GB1 spectrum was measured with a maximum ¹⁵N evolution time of 10 ms.

Spectral Processing, Numerical Simulations and Structure Calculations

NMR spectra were processed in the TopSpin software (Bruker Biospin) using Gaussian window functions with parameters of LB = -15 Hz and GB = 0.05. Numerical simulations were conducted using the SpinEvolution software package ⁴⁵. Atomic coordinates and ¹³C isotropic chemical shifts for a fragment containing the Met Ca, Cβ, C', N, H^N, Ha, Hβ1, and Hβ2 from the SSNMR structure of f-MLF ⁴⁶ were used to create an eight-spin system.

Explicit coordinates and chemical shift anisotropy (CSA) parameters used in the simulations are listed in Table S1^{32, 47}. Simulated data was processed and analyzed in MATLAB (MathWorks, Natick, MA). GB1 structure calculations were performed using the CYANA software⁴⁸. A total of 100 conformers were created, each with 35,000 (ϕ , ψ) torsion angle steps. The ten conformers with the lowest target function were included in the final ensemble.

Results and Discussion

Fig. 1 shows the ^{15}N - ^{13}C CP schemes incorporated into 2D correlation pulse sequences. The most commonly used ^{13}C , ^{15}N and ^1H rf field strengths for the ^{15}N - ^{13}C CP period are indicated. The SPECIFICCP sequence requires CW irradiation on all three channels, including strong ^1H decoupling, to achieve efficient ^{15}N - ^{13}C transfer. The PAINCP pulse sequence uses moderate ^1H rf fields but requires stronger rf fields for ^{15}N and ^{13}C compared to SPECIFICCP. The RESPIRATIONCP sequence requires strong ^1H decoupling, but only employs ^{15}N pulses at the rotor echoes while the ^{13}C channel employs both rotor-echo pulses and CW irradiation at a field strength of twice the MAS frequency. In comparison, the PERSPIRATIONCP sequence uses the lowest rf energy: the ^1H irradiation is slightly milder than PAINCP and only rotor-echo pulses are applied on the ^{13}C and ^{15}N channels.

Since PERSPIRATIONCP scheme is an amalgam of the PAINCP and RESPIRATIONCP schemes, we first summarize the mechanisms of polarization transfer by these two techniques. The RESPIRATIONCP first-order average Hamiltonian under the condition of ^{13}C and ^{15}N 90° rotor-echo pulses and ^{13}C recoupling field strength of $\omega_1 = 2\omega_r$ is $\bar{H}_D^{(1)} = (4b_{IS} / 3\sqrt{2})\sin 2\beta \cos \gamma (\mathbf{I}_y \mathbf{S}_z - \mathbf{I}_z \mathbf{S}_y) + (b_{IS} / 2\sqrt{2})\sin^2 \beta \cos 2\gamma (\mathbf{I}_y \mathbf{S}_y + \mathbf{I}_z \mathbf{S}_z)$, where b_{IS} is the dipolar coupling constant and (β , γ) specify the polar and azimuthal angles of the ^{15}N - ^{13}C dipolar vector in the rotor-fixed frame³⁷. The coefficients in front of the two spin-operator terms correspond to the first- and second-order Fourier components of the dipolar couplings, thus RESPIRATIONCP provides more complete orientational averaging of the dipolar coupling than γ -encoded recoupling sequences and hence is more efficient⁴⁹. In comparison, zero-quantum PAINCP relies on trilinear terms of the form $N C^\pm H_z$ for ^{15}N - ^{13}C polarization transfer³²⁻³³, while the PAR mechanism that is also active during the CP contact time involves a similar trilinear term $C_1 C_2^\pm H_z$ for ^{13}C - ^{13}C coherence transfer³⁴. By using second-order cross terms between the ^{13}C - ^1H and ^{15}N - ^1H dipolar couplings instead of direct ^{15}N - ^{13}C and ^{13}C - ^{13}C dipolar couplings, PAINCP allows polarization transfer over longer distances, and significantly avoids the dipolar truncation problem, which is present in first-order homonuclear recoupling sequences due to non-commuting terms in the effective Hamiltonians⁵⁰. Dipolar truncation can also be present in heteronuclear recoupling sequences with longitudinal average Hamiltonians such as REDOR, where non-commuting terms arise when the recoupling pulses take up significant fractions of the rotor period.

PERSPIRATIONCP results in efficient long-range ^{15}N - ^{13}C transfer using mild rf fields

We compared the performance of these four ^{15}N - ^{13}C CP techniques using uniformly ^{13}C , ^{15}N -labeled tripeptide formyl-Met-Leu-Phe (f-MLF)²⁷ and GB1⁴⁰ (Fig. 2). We monitor

two quantitative features of the spectra: the overall sensitivity of a ^{13}C spectrum and the relative intensities of the $\text{C}\alpha$, CO , and aliphatic sidechain regions. We measure the overall sensitivity as the integrated intensities of the whole ^{15}N -transferred ^{13}C spectrum relative to the integrated intensity of a ^1H - ^{13}C CP spectrum. On the anhydrous f-MLF, the four ^{15}N - ^{13}C CP sequences gave sensitivities of 2-7% relative to ^1H - ^{13}C CP (Fig. 2a-e). This percentage range is expected based on the typical ^1H , ^{13}C and ^{15}N spin densities in proteins. f-MLF contains 32 protons, 3 nitrogens, and 20 carbons. Thus, the ^{15}N -transferred ^{13}C magnetization, which results exclusively from the three ^{15}N spins, carry $3 \cdot 32 / (32 + 3) = 2.7$ units of ^1H magnetization. Assuming equal distribution of the magnetization between ^{15}N and ^{13}C , the total ^{13}C magnetization after ^{15}N - ^{13}C CP should then be 1.35 units of ^1H magnetization. In comparison, the ^{13}C magnetization after ^1H - ^{13}C CP results from all 32 protons, which amounts to $20 \cdot 32 / (32 + 20) = 12.3$ units of ^1H magnetization. Therefore, the maximum theoretical efficiency of ^{15}N - ^{13}C CP is expected to be ~11% that of ^1H - ^{13}C CP when normalized by the full ^{13}C spectral intensity. This differs from the commonly used normalization by $\text{C}\alpha$ intensities, which gives higher numerical values of efficiencies in the literature.

SPECIFICCP combined with 60 ms ^{13}C spin diffusion by CORD⁵¹ gave rise to the highest overall sensitivity among the four CP sequences for f-MLF. This high sensitivity can be attributed to the strong ^1H decoupling field of 100 kHz, which was more than 3-times larger than the ^{13}C and ^{15}N rf field strengths. In addition, the relatively short ^{15}N - ^{13}C CP contact time of 1.5 ms minimized ^{13}C and ^{15}N $T_{1\rho}$ relaxation while still being sufficient for one-bond transfer in $\text{N}-\text{C}\alpha$ and $\text{N}-\text{CO}$ pairs. The other three CP schemes gave lower total sensitivities, which were 2.1-3.8% for PAINCP and 3.9-6.0% for PERSPIRATIONCP and RESPIRATIONCP .

While SPECIFICCP has high total sensitivity, polarization transfer to sidechains carbons does not occur without ^{13}C mixing. When a CORD mixing time of 60 ms was added, the spectrum shows decreasing intensities toward the methyl region (Fig. 2d), consistent with the known attenuation of ^{13}C spin diffusion with increasing chemical shift difference. RESPIRATIONCP gave the lowest polarization transfer to sidechains among the four CP methods (Fig. 2c). PAINCP gave higher magnetization transfer to sidechains, but the performance is significantly worsened at lower ^{13}C , ^{15}N and ^1H rf field strengths of 25, 25, and 48 kHz, respectively (Fig. 2b). In comparison, PERSPIRATIONCP exhibits efficient magnetization transfer to sidechain carbons at both strong and weak rf powers as well as higher overall sensitivity compared to PAINCP .

These ^{15}N - ^{13}C polarization transfer trends are qualitatively reproduced in hydrated GB1 (Fig. 2f-j), whose ^{13}C and ^{15}N $T_{1\rho}$ relaxation times are shorter than those of anhydrous f-MLF^{27, 46}. We used the same ^{15}N and ^{13}C rf field strengths of 35 kHz, a contact time of 5 ms, and a ^{13}C carrier frequency of 42 ppm for all but the SPECIFICCP experiment. PERSPIRATIONCP gave higher-sensitivity spectra and more efficient polarization transfer to sidechain carbons than RESPIRATIONCP and SPECIFICCP followed by ^{13}C mixing. PAINCP at similar rf field strengths gave the highest polarization transfer to the sidechains, but had low overall sensitivity. PERSPIRATIONCP only underperformed in one aspect, which is the transfer to Thr $\text{C}\beta$ and $\text{C}\gamma$ and to Ala $\text{C}\beta$, suggesting that the sequence has a stronger dependence on

resonance offset than ^{15}N - ^{13}C PAINCP. The carrier dependence of these CP experiments is further investigated below by simulations.

The f-MLF data also show how these ^{15}N - ^{13}C CP sequences depend on the rf field strengths. RESPIRATIONCP showed higher sensitivity under low-power (25 kHz) rotor-echo pulses than high-power (50 kHz) pulses, indicating that CP leakage from ^{13}C to ^1H under high-power irradiation is detrimental to ^{15}N - ^{13}C polarization transfer. For PAINCP, low-power irradiation resulted in more band-selective polarization transfer as well as lower overall sensitivity (55%) than high-power irradiation. We attribute this finding to faster ^{13}C and ^{15}N $T_{1\rho}$ relaxation under weaker spin lock fields as well as narrower matching conditions. PERSPIRATIONCP showed efficient polarization transfer to sidechains under both low-power and high-power irradiation, but the high-power condition gave 40% higher intensity.

Fig. 3 plots the measured ^{15}N - ^{13}C polarization transfer intensities for the Met signals of f-MLF as a function of contact time for PERSPIRATIONCP, PAINCP, and RESPIRATIONCP. The three sequences show distinct dependences on internuclear distances, rf field strengths, and contact times for maximum polarization transfer. RESPIRATIONCP shows the strongest distance dependence: for example, one-bond N-C α and N-CO transfer reached maximum intensity at ~4 ms, while two-bond N-C β transfer reached maximum at 6-7 ms. PAINCP exhibits a more moderate distance dependence. Under the strong rf condition, maximum one-bond transfer is achieved at ~1.5 ms while two-bond N-C β transfer reached a plateau at ~3 ms. Weak rf fields significantly suppressed the extent of polarization transfer for PAINCP. PERSPIRATIONCP shows the weakest distance dependence: all carbons reached similar magnitudes of maximum transfer and at similar contact times of about 6-8 ms.

To further understand the spin dynamics of PERSPIRATIONCP, we simulated the CP buildup curves using an eight-spin system that corresponds to the Met residue in f-MLF (Fig. 4). These simulations reproduce the experimentally measured polarization transfer profiles, and moreover give insight into the mechanism of transfer. PERSPIRATIONCP has relatively uniform transfer rates for all carbons. For both PERSPIRATIONCP and PAINCP, the polarization transfer is unchanged when ^{15}N - ^{13}C dipolar couplings are turned off, but no transfer occurs when ^{13}C - ^1H and ^{15}N - ^1H dipolar couplings are turned off (Fig. 4c, d). Therefore, PERSPIRATIONCP, like PAINCP^{32, 34}, operates through a ^1H -assisted mechanism. The main difference between these two sequences is that when ^{13}C - ^{13}C and ^1H - ^1H dipolar couplings are turned off, N-C β transfer became much more efficient in the PERSPIRATIONCP experiment while unchanged in the PAINCP experiment. In contrast, RESPIRATIONCP transfer shows strong distance dependence, and the transfer is suppressed when ^{15}N - ^{13}C dipolar couplings are turned off but speeded up when ^1H - ^{15}N and ^1H - ^{13}C dipolar couplings are turned off. These results confirm the first-order nature of the RESPIRATIONCP sequence.

PERSPIRATIONCP is optimal with 90° pulses and with the ^{13}C carrier in the aliphatic region

RESPIRATIONCP is relatively insensitive to the pulse flip angles and allows 5-175° pulses to be used on the rotor echoes. To investigate how PERSPIRATIONCP depends on the pulse flip angles, we varied the ^{13}C and ^{15}N pulse lengths from 60° to 360° using 50 kHz rf fields on f-MLF. We also tested longer pulses up to a full rotor period under 20 kHz MAS, which corresponds to a 900° flip angle. Similar tests were conducted for GB1, but using an rf field

of 35 kHz for both ^{13}C and ^{15}N and varying the pulse flip angles from 60° up to a rotor period. Fig. 5 shows that the highest polarization transfer is achieved for 90° pulses. Slightly larger flip angles (120°) enhance the signals of aliphatic sidechain peaks such as Leu C δ_2 (19.6 ppm) in f-MLF and Thr C γ (16-21 ppm) in GB1 while slightly smaller flip angles (60°) enhance the signal of carbonyl peaks. 360° pulses quenched polarization transfer, while 180° pulses performed poorly for GB1 but still gave some intensities for f-MLF. Numerical simulations (Fig. 6a, Fig. S1) confirm these observations, showing maximum polarization transfer at 90° and vanishing transfer at integer multiples of 180° . Simulated pulses slightly shorter than 90° favor transfer to the carbonyl peak at 172 ppm, while pulses slightly longer than 90° favor aliphatic sidechain peaks. Because a significant range of flip angles can yield good overall transfer ($75\text{--}120^\circ$), $\text{PERSPIRATION}_{\text{CP}}$ is insensitive to B_1 inhomogeneity. Finally, the width of the null is wider at 360° than at 180° and 540° , consistent with the experimental data. Further simulations indicate that it is the flip angle of the rotor-echo pulses rather than rf field matching that dictates polarization transfer (Fig. S2). Therefore, there is considerable freedom in setting up experiments when the ^{15}N rf field strengths are more limited than ^{13}C , as is often the case in triple-resonance MAS probes.

The isotropic chemical shift evolution during the delays in the $\text{PERSPIRATION}_{\text{CP}}$ sequence is expected to cause a stronger offset dependence than the spin-lock based PAIN_{CP} experiment. Indeed, Fig. 7a shows that $\text{PERSPIRATION}_{\text{CP}}$ gives optimal polarization transfer when the ^{13}C carrier frequency is centered in the aliphatic region (near the Met C γ peak for f-MLF) but almost no transfer when the carrier is placed at 100 ppm. Numerical simulations confirm these experimental results (Fig. 6b). In comparison, PAIN_{CP} gives optimal polarization transfer with the ^{13}C carrier at 100 ppm (Fig. 7b, 6c). However, the carrier dependence of PAIN_{CP} is sensitive to the rf field strengths. With $^{13}\text{C}/^{15}\text{N}/^1\text{H}$ rf field strengths of 48.9/50/63 kHz, the ^{13}C carrier dependence differ significantly from the carrier dependence when the ^{13}C rf field is slightly changed to 50 kHz or 49.5 kHz (Fig. S3). For example, with 50 kHz ^{13}C pulses, a carrier frequency of 100 ppm results in weak transfer to C α and almost no transfer to C β or CO.

To better understand the carrier dependence of $\text{PERSPIRATION}_{\text{CP}}$, we consider the first-order average Hamiltonian of the experiment. Approximating the ^{13}C and ^{15}N rotor-echo 90° pulses as δ -function pulses, the various nuclear spin Hamiltonians in the interaction frame of the ^{13}C and ^{15}N pulses are:

$$\begin{aligned}
 H_{t_0 \rightarrow t_1} &= \omega_{\text{NC}} 2N_z C_z + \omega_{\text{CH}} 2C_z H_z + \omega_{\text{NH}} 2N_z H_z + \omega_{\text{C}} C_z + \omega_{\text{N}} N_z + \omega_{\text{H}} H_z + \omega_{1,\text{H}} H_x \\
 H_{t_1 \rightarrow t_2} &= \omega_{\text{NC}} 2N_y C_y + \omega_{\text{CH}} 2C_y H_z + \omega_{\text{NH}} 2N_y H_z + \omega_{\text{C}} C_y + \omega_{\text{N}} N_y + \omega_{\text{H}} H_z + \omega_{1,\text{H}} H_x \\
 H_{t_2 \rightarrow t_3} &= \omega_{\text{NC}} 2N_z C_z - \omega_{\text{CH}} 2C_z H_z - \omega_{\text{NH}} 2N_z H_z - \omega_{\text{C}} C_z - \omega_{\text{N}} N_z + \omega_{\text{H}} H_z + \omega_{1,\text{H}} H_x \\
 H_{t_3 \rightarrow t_4} &= \omega_{\text{NC}} 2N_y C_y - \omega_{\text{CH}} 2C_y H_z - \omega_{\text{NH}} 2N_y H_z - \omega_{\text{C}} C_y - \omega_{\text{N}} N_y + \omega_{\text{H}} H_z + \omega_{1,\text{H}} H_x
 \end{aligned} \tag{1}$$

The spin interactions in the above Hamiltonians correspond to, from left to right, ^{15}N - ^{13}C dipolar coupling, ^{13}C - ^1H dipolar coupling, ^{15}N - ^1H dipolar coupling, ^{13}C chemical shift, ^{15}N chemical shift, ^1H chemical shift, and the ^1H rf Hamiltonian. The pulse sequence is cyclic

for the least common multiple of $4\tau_r$ and $2\pi/\omega_{1,H}$. When ^{13}C and ^{15}N spins are on resonance, the first-order average Hamiltonians for all interactions, calculated in the interaction frame of the ^1H rf pulse, can be shown to be zero ⁵²:

$$\overline{\tilde{H}}_{D,NC}^{(0)} = \overline{\tilde{H}}_{D,CH}^{(0)} = \overline{\tilde{H}}_{D,NH}^{(0)} = \overline{\tilde{H}}_{CS,C}^{(0)} = \overline{\tilde{H}}_{CS,N}^{(0)} = \overline{\tilde{H}}_{CS,H}^{(0)} = 0 \quad (2)$$

This averaging of the first-order average Hamiltonians is expected for a second-order recoupling technique. However, ^{13}C chemical shift offset interferes with this averaging. For example, for a 5-kHz off-resonance ^{13}C spin, at 20 kHz MAS, after one rotor period, the ^{13}C spin will have precessed by 90° , thus making the three spin interactions that are linear with the ^{13}C spin operator non-vanishing. On the other hand, ^{13}C spins that are 20 kHz off resonance will have precessed for 360° after one rotor period and thus will behave as if they were on resonance. Therefore, the PERSPIRATION_{CP} sequence should exhibit an offset dependence that is related to the MAS frequency. This is seen in both experiments and simulations. In the f-MLF and GB1 PERSPIRATION_{CP} spectra in Fig. 2a, f, which were measured with the ^{13}C carrier at ~40 ppm, near-resonance aliphatic ^{13}C signals as well as ^{13}CO signals that are ~130 ppm or ~20 kHz off resonance both show high intensities, while the aromatic ^{13}C signals, which are ~90 ppm (or 13.5 kHz) off resonance, are suppressed. For the same reason, Thr C β and C γ 2 and Ala C β signals, which lie at the edges of the aliphatic region, ~3.8 kHz off resonance, show lower transfer efficiencies than near-resonance aliphatic ^{13}C signals. Fig. 6b shows simulated ^{15}N - ^{13}C transfer efficiencies as a function of the ^{13}C carrier frequency. With the carrier in the aliphatic region, efficient transfer to ^{13}C signals within ± 3 kHz of the carrier and to the CO signals is observed. An identical band of carrier frequencies that exhibit high transfer efficiencies is found in the carbonyl region, which is ~20 kHz away from the C α and C β chemical shifts on a 600 MHz spectrometer.

Inter-residue cross peaks are readily observed in 2D ^{15}N - ^{13}C PERSPIRATION_{CP} correlation spectra

We compared the 2D ^{15}N - ^{13}C correlation spectra of f-MLF (Fig. 8) measured using the four CP experiments. SPECIFIC_{CP} followed by 60 ms ^{13}C CORD spin diffusion ⁵¹ gave rise to the expected intra-residue and inter-residue cross peaks in the 2D NCACX and NCOCX spectra, but with this mixing time, the cross peak intensities of C γ and other carbons down the sidechains drop off significantly (Fig. 8a). RESPIRATION_{CP} gave strong one-bond N-C α and N-CO cross peaks but weaker cross peaks to sidechain carbons than SPECIFIC_{CP}, consistent with the exclusive dependence of RESPIRATION_{CP} on ^{15}N - ^{13}C dipolar couplings. The 2 ms PAIN_{CP} spectrum showed stronger long-range intra- and inter-residue cross peak intensities than both SPECIFIC_{CP} and RESPIRATION_{CP}, consistent with its proton-assisted recoupling mechanism. In particular, cross peaks between the isolated Met C ϵ and the Met and Phe ^{15}N are observed (Fig. 8c). The 5 ms PERSPIRATION_{CP} 2D spectrum showed higher cross peak intensities for C β and C γ carbons than the 2 ms PAIN_{CP} spectrum, even though the total rf energy in 5 ms PERSPIRATION_{CP} is only a third of the rf energy in 2 ms PAIN_{CP} (Table 1). The only cross peaks that are weaker in the PERSPIRATION_{CP} spectrum are the Met C ϵ cross peaks, which can be attributed to the offset dependence of PERSPIRATION_{CP}. With a contact

time of 9 ms, the $\text{PERSPIRATION}_{\text{CP}}$ spectral intensities are nearly fully equilibrated and are much higher than the PAIN_{CP} intensities.

The 2D spectra of GB1 (Fig. 9, 10a) reproduce the trends observed for f-MLF. The 5 ms $\text{PERSPIRATION}_{\text{CP}}$ spectrum gives more long-range ^{15}N - ^{13}C cross peaks than the sum of the NCACX and NCOCX 2D spectra measured with 100 ms ^{13}C spin diffusion mixing. For example, A48N-K50C δ and F30N-V29C β cross peaks, which correspond to 5 Å distances, are observed in the $\text{PERSPIRATION}_{\text{CP}}$ spectrum but are absent in the sum of the 2D NCACX and NCOCX spectrum (Fig. 9d). The 5.0 ms 2D $\text{PERSPIRATION}_{\text{CP}}$ spectrum used rotor-echo pulses of 112.5° rather than 90° to broaden the bandwidth of polarization transfer in the aliphatic region and to enhance the Thr C β (72-62 ppm), Thr C γ_2 and Ala C β (21-16 ppm) intensities. Increasing the $\text{PERSPIRATION}_{\text{CP}}$ transfer time to 10 ms gave rise to additional cross peaks (Fig. 10a), many of which correspond to longer distances than in the 5 ms spectrum. For example, cross peaks assigned to L12N-N8C α , T16N-I6C β , V54N-L5C α , and E56N-K10C ϵ correspond to distances of 6.5 – 7.0 Å.

$\text{PERSPIRATION}_{\text{CP}}$ yields many distance constraints for protein structure determination

To investigate the utility of $\text{PERSPIRATION}_{\text{CP}}$ for protein structure determination, we calculated the structure of GB1 using only distance restraints from the 5 ms and 10 ms $\text{PERSPIRATION}_{\text{CP}}$ 2D ^{15}N - ^{13}C correlation spectra. A total of 32 medium- and long-range distance constraints were assigned from these two spectra, along with 81 sequential cross peaks and 109 intra-residue cross peaks (Table 2). These distance constraints supplement the (ϕ , ψ) torsion angles predicted from assigned chemical shifts⁵³. We set the upper distance limits to 5 Å for all cross peaks detected in the 5 ms $\text{PERSPIRATION}_{\text{CP}}$ spectrum and 7 Å for additional cross peaks observed in the 10 ms spectrum. These upper limits are estimated based on comparison with the PAIN_{CP} data in the literature³²⁻³³ and by calibrating with intra-residue ^{15}N cross peaks with sidechain carbons with approximately known distances. The ensemble of ten lowest-energy structures calculated using CYANA shows a backbone RMSD of 1.4±0.3 Å and a heavy-atom RMSD of 2.1±0.3 Å. The structure correctly recapitulates the known three-dimensional fold of GB1 and the relative positions and orientations of the α -helix, β -strands, and loops (Fig. 10b). Compared to the high-resolution SSNMR structure of GB1 (PDB: 2LGI)⁵⁴ (Fig. 10c), the $\text{PERSPIRATION}_{\text{CP}}$ structure has a backbone RMSD of 2.0 Å and a heavy-atom RMSD of 3.0 Å. This agreement is remarkably good considering that we only used the ^{15}N - ^{13}C distance restraints from two $\text{PERSPIRATION}_{\text{CP}}$ spectra. The main deviation of the $\text{PERSPIRATION}_{\text{CP}}$ structure from the high-resolution structure is the separation between β -strands 1 and 2, which is a direct consequence of the relative sparseness of ^{15}N - ^{13}C constraints between these two strands. Thus, the resolution and accuracy of the calculated structure can be easily improved by measuring longer mixing-time ^{15}N - ^{13}C $\text{PERSPIRATION}_{\text{CP}}$ spectra, and by adding ^{13}C - ^{13}C distance restraints, which are readily measurable from 2D ^{13}C - ^{13}C correlation experiments. In arriving at this GB1 structure, we also tested alternative distance upper limits for the two mixing times, such as 6 Å and 8 Å, or 4 Å and 6 Å. These combinations gave structures with either larger RMSDs for the ensemble or larger deviations from the high-resolution structure. The structure calculated with the 5 Å and 7 Å upper limits has the lowest RMSDs for the ensemble, the best agreement with the high-resolution structure, and the lowest

constraint violation. These comparisons thus calibrate the maximum ^{15}N - ^{13}C distances observable with 5 ms and 10 ms $^{\text{PERSPIRATION}}\text{CP}$ to be 5 Å and 7 Å.

Conclusions

The above experiments and simulations show that $^{\text{PERSPIRATION}}\text{CP}$ is an rf-efficient pulsed variant of $^{\text{PAIN}}\text{CP}$ that operates by a third-spin proton-assisted recoupling mechanism. Both techniques combine ^{15}N - ^{13}C polarization transfer with ^{13}C - ^{13}C mixing, and have an attenuated dependence on ^{15}N - ^{13}C distances compared to first-order recoupling sequences such as $^{\text{SPECIFIC}}\text{CP}$ and $^{\text{RESPIRATION}}\text{CP}$. While this weaker dependence decreases the accuracy of individual distances, it massively increases the number of observable cross peaks, thus ultimately improving the ability for structure determination. Because $^{\text{PERSPIRATION}}\text{CP}$ requires only rotor-echo 90° pulses on ^{15}N and ^{13}C and moderate CW irradiation on ^1H , it uses 3-7 times less rf energy compared to $^{\text{PAIN}}\text{CP}$ and $^{\text{RESPIRATION}}\text{CP}$, and up to 2-fold less rf energy than $^{\text{SPECIFIC}}\text{CP}$ (Table 1). Even a 9 ms $^{\text{PERSPIRATION}}\text{CP}$ experiment uses only 60% of the rf energy compared to 2 ms $^{\text{PAIN}}\text{CP}$ for similar $^{13}\text{C}/^{15}\text{N}/^1\text{H}$ rf field strengths of ~50/50/60 kHz. In addition to rf efficiency, $^{\text{PERSPIRATION}}\text{CP}$ shows higher ^{15}N - ^{13}C polarization transfer efficiency than $^{\text{PAIN}}\text{CP}$, especially at low rf field strengths. The rotor-echo pulse flip angles in $^{\text{PERSPIRATION}}\text{CP}$ can deviate from 90° by a moderate amount to compensate for the increased offset dependence of the technique due to the windowed delays.

2D ^{15}N - ^{13}C $^{\text{PERSPIRATION}}\text{CP}$ spectra of GB1 show higher intensities than the sum of the NCACX and NCOCX spectra measured with $^{\text{SPECIFIC}}\text{CP}$. The distance constraints measured from only two $^{\text{PERSPIRATION}}\text{CP}$ spectra were sufficient to yield a GB1 structure with only a 2.0 Å backbone RMSD from the high-resolution structure. Therefore, $^{\text{PERSPIRATION}}\text{CP}$ is a robust and rf-efficient alternative to the existing CP pulse sequences for resonance assignment and structure determination of proteins, and should be useful for measuring long-range intramolecular ^{15}N - ^{13}C distance restraints in uniformly labeled proteins and long-range intermolecular distance restraints in mixed labeled proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

^{15}N - ^{13}C polarization transfer pulse sequences compared in this study. (a) ^{15}N - ^{13}C SPECIFIC_{CP} followed by ^{13}C - ^{13}C spin diffusion by CORD. (b) PAIN_{CP}. (c) RESPIRATION_{CP}, where simultaneous short ^{13}C and ^{15}N pulses at rotor echoes are interspersed with phase-alternating ^{13}C recoupling pulses during the rotor period with $\omega_1 = 2\omega_r$. (d) PERSPIRATION_{CP} sequence, where simultaneous ^{13}C and ^{15}N pulses are applied on the rotor echoes. Typical rf field strengths for the ^{15}N - ^{13}C CP block that lead to high-efficiency polarization transfer are indicated.

Figure 2.

¹³C spectra of f-MLF (a-e) and GB1 (f-j) measured using difference CP schemes. All spectra were measured under 20 kHz MAS. Indicated for each spectrum are the ¹³C/¹⁵N/¹H rf field strengths for the CP period, the ¹⁵N-¹³C CP contact time, ¹³C carrier frequencies (arrows), and relative total spectral intensities. (a) PERSPIRATION_{CP} spectra of f-MLF, measured with ¹³C/¹⁵N/¹H rf fields of 50/50/57 kHz and 25/25/38 kHz. (b) PAIN_{CP} spectra of f-MLF, measured with two strong and weak rf field strengths at two different ¹³C carrier frequencies. (c) RESPIRATION_{CP} spectra of f-MLF. (d) N-Cα SPECIFIC_{CP} spectra of f-MLF, measured with rf fields of 10/30/100 kHz with and without ¹³C-¹³C mixing. (e) ¹H-¹³C CP spectrum measured using 70-100% ramp on ¹³C for 0.75 ms. (f) ¹⁵N-¹³C PERSPIRATION_{CP}

spectrum of GB1. **(g)** ^{15}N - ^{13}C PAIN CP spectrum of GB1. **(h)** ^{15}N - ^{13}C RESPIRATION CP spectrum of GB1. **(i)** N-C α SPECIFIC CP spectra of GB1 with and without ^{13}C - ^{13}C mixing. **(j)** ^1H - ^{13}C CP spectrum of GB1 using 70-100% ramp on ^{13}C for 0.75 ms.

Figure 3. Experimental ^{15}N - ^{13}C polarization transfer curves of f-MLF as a function of CP contact time. (a) $\text{PERSPIRATION}_{\text{CP}}$. (b) PAIN_{CP} . (c) $\text{RESPIRATION}_{\text{CP}}$. Left column shows data measured using low rf field strengths while the right column shows data measured using strong rf fields. The ^{13}C carrier frequency was 37.5 ppm for the $\text{PERSPIRATION}_{\text{CP}}$ data and the low-power PAIN_{CP} data, and 100 ppm for the other data. The ^{13}C , ^{15}N and ^1H rf field strengths during ^{15}N - ^{13}C CP are given in the individual panels.

Figure 4.

Simulated ^{15}N - ^{13}C polarization transfer efficiencies as a function of contact time for PERSPIRATION_{CP}, PAIN_{CP}, and RESPIRATION_{CP} sequences. (a) An eight-spin system containing $C\alpha$, $C\beta$, C' , N , H_N , $H_{C\alpha}$, $H_{C\beta 1}$, and $H_{C\beta 2}$ used in the simulations and comparison of N - $C\alpha$ and N - $C\beta$ CP transfer curves for the three CP sequences. (b-d) Dependence of the N - $C\alpha$ and N - $C\beta$ polarization transfer efficiency on C-N, N-H, C-H dipolar couplings and C-C and H-H dipolar couplings for the (b) PERSPIRATION_{CP}, (c) PAIN_{CP}, and (d) RESPIRATION_{CP} experiments. PERSPIRATION_{CP} and PAIN_{CP} do not require ^{15}N - ^{13}C dipolar couplings but require ^{13}C - ^1H and ^{15}N - ^1H dipolar couplings.

Figure 5. Dependence of $^{\text{PERSPIRATION}}\text{CP}$ polarization transfer on the ^{13}C and ^{15}N pulse flip angles. **(a)** f-MLF data. **(b)** GB1 data. Both the flip angle and the pulse fraction of the rotor period are indicated. The $^{13}\text{C}/^{15}\text{N}/^1\text{H}$ rf field strengths for ^{15}N - ^{13}C CP were 50/50/57 kHz for MLF and 35/35/54 kHz for GB1. The ^{13}C carrier frequency was 37.5 ppm for f-MLF and 42 ppm for GB1.

Figure 6.

Simulated dependences of ^{15}N - ^{13}C polarization transfer efficiencies on pulse flip angle and ^{13}C carrier frequency. **(a)** PERSPIRATION_{CP} efficiency as a function of the pulse flip angle.

The fraction of the rotor period occupied by the pulses is shown on the top x-axis. **(b)**

PERSPIRATION_{CP} efficiency as a function of ^{13}C carrier frequency. **(c)** PAIN_{CP} efficiency as a function of ^{13}C carrier frequency. All simulations used a 5 ms CP contact time.

Figure 7.

Dependence of ^{13}C NMR spectra on the ^{13}C carrier frequency, which is denoted by an arrow below each spectrum. (a) ^{13}C NMR spectra. (b) ^{13}C NMR spectra. The ^{15}N carrier frequency was fixed at 118.5 ppm.

Figure 8.

2D ^{15}N - ^{13}C correlation spectra of f-MLF measured using the four CP schemes. **(a)** SPECIFICCP NACX and NCOCX spectra, measured with a 1.5 ms contact time, $^{13}\text{C}/^{15}\text{N}/^1\text{H}$ rf field strengths of 10/30/100 kHz and 60 ms ^{13}C - ^{13}C mixing. **(b)** RESPIRATIONCP spectrum, measured with a 5 ms contact time and $^{13}\text{C}/^{15}\text{N}/^1\text{H}$ field strengths of 50/50/100 kHz. **(c)** PAINCP spectrum, measured with a 2 ms contact time and $^{13}\text{C}/^{15}\text{N}/^1\text{H}$ field strengths of 50/50/63 kHz. **(d)** PERSPIRATIONCP spectra, measured with contact times of 5 ms and 9 ms and $^{13}\text{C}/^{15}\text{N}/^1\text{H}$ rf field strengths of 50/50/57 kHz.

Figure 9.

$2D$ ^{15}N - ^{13}C correlation spectra of GB1 measured using (a) $2D$ NCACX (blue) and NCOCX (red) experiments and (b) $2D$ NCCX PERSPIRATION_{CP} experiment with a 5 ms contact time. (c) Representative ^{15}N cross sections from the three spectra. The sum of the $2D$ NCACX and NCOCX spectra (green) has lower intensities than the PERSPIRATION_{CP} spectrum after taking into account the 2-fold difference in the number of scans. (d) Selected region of the $2D$ PERSPIRATION_{CP} spectra and the sum of the SPECIFIC_{CP} spectra. The PERSPIRATION_{CP} spectrum shows several intra- and inter-residue cross peaks for ~ 5 Å distances, which are not observed in the sum of the two SPECIFIC_{CP} spectra.

Figure 10.

(a) Selected region from the 10 ms $\text{PERSPIRATION}_{\text{CP}}$ ^{15}N - ^{13}C correlation spectra of GB1. Numerous medium and long-range cross peaks are observed. (b) GB1 structure calculated using ^{15}N - ^{13}C distance constraints from the $\text{PERSPIRATION}_{\text{CP}}$ spectra. (c) High-resolution SSNMR structure of GB1 (PDB: 2LGI). Dashed lines indicate the residue pairs for which medium and long-range cross peaks were observed in the $\text{PERSPIRATION}_{\text{CP}}$ spectra.

Table 1.

Contact times, rf field strengths and rf powers used for four ^{15}N - ^{13}C CP experiments.

Pulse sequence	Sample	Mixing time (ms)	Time-averaged rf fields (kHz)			Total rf energy (mJ)	Relative rf energy
			^{13}C	^{15}N	^1H		
PERSPIRATION _{CP}	f-MLF	5	5	5	57	248	1.0
PAIN _{CP}		2	50	50	63	717	2.9
SPECIFIC _{CP} + CORD		1.5, 60	10	30	100, 13.3	260	1.0
RESPIRATION _{CP}		5	45	5	100	655	2.6
PERSPIRATION _{CP}	f-MLF	5	5	5	38	76	1.0
PAIN _{CP}		2	25	25	48	204	2.7
RESPIRATION _{CP}		5	45	5	100	525	7.0
PERSPIRATION _{CP}		5	5	5	54	315	1.0
PAIN _{CP}	GB1	5	35	35	54	1245	4.0
SPECIFIC _{CP} + CORD		1.5, 100	12.5	32.5	90, 13.3	593	1.9
RESPIRATION _{CP}		5	45	5	90	874	2.8

Table 2.

^{15}N - ^{13}C distance constraints obtained from $^{\text{PERSPIRATION}}\text{CP}$ spectra used for GB1 structure calculation.

$^{\text{PERSPIRATION}}\text{CP}$ time (ms)	Intra-residue constraints	Sequential constraints ($ i-j = 1$)	Medium-Range constraints ($2 \leq i-j \leq 4$)	Long-Range Constraints ($ i-j \geq 5$)
5	101	69	9	8
10	8	12	2	13