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^{13}C Nuclear Overhauser Polarization−Magic-Angle Spinning Nuclear Magnetic Resonance Spectroscopy in Uniformly ^{13}C-Labeled Solid Proteins

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Abstract: A recently introduced ^{13}C polarization technique based on the nuclear Overhauser effect in rotating solid (nuclear Overhauser polarization−magic-angle spinning, NOP-MAS) (Takegoshi, K.; Terao, T. *J. Chem. Phys.* **2002**, *117*, 1700−1707) is applied to uniformly ^{13}C, ^{15}N-labeled proteins. NOP enhancement factors per scan of 1.5∼2.0 are obtained, while that by cross polarization (CP) is less than 1.0. We show that uniform enhancement of all ^{13}C signals by CP is difficult to attain, while it is easily achieved by NOP, thus enabling quantitative comparison of signal intensities. NOP is easy to carry out under fast MAS and works well even for somewhat mobile molecules, for which CP does not work. Moreover, in labeled protein samples containing nonlabeled additives, NOP can eliminate the latter signals. For these features, NOP is superior to CP in many uniformly ^{13}C labeled proteins.

Introduction

Cross polarization (CP) has been used for a long time as a prerequisite technique for improving the sensitivity of dilute and/or low γ spin (S) NMR in solids.[1,2] CP enables us to acquire a signal with a repetition time comparable to the spin−lattice relaxation time (T_1) of the ^{1}H spins (I), which is usually much shorter than those of the S spins, and also to enhance the magnetization of dilute S spins by $\sim\gamma_I/\gamma_S$ compared to the thermal equilibrium value. CP has been applied also to uniformly ^{13}C-labeled samples of peptides, proteins, and the like. In this work, however, we show that the advantage of CP becomes less appealing for uniformly ^{13}C-labeled proteins. This is partly because the theoretical maximum gain is reduced to be ∼2.4 for a uniformly ^{13}C-labeled protein due to the low proton-to-carbon ratio N_I/N_S, which is 1.65 on the average for the 20 standard amino acid residues. Moreover, mobility in protein molecules may reduce the spin−lattice relaxation times in the rotating frame ($T_{1\rho}^S$ and $T_{1\rho}^I$), leading to a further decrease of the enhancement factor. It is further pointed out in this work that uniform enhancement is difficult for CP, particularly in proteins due to the differences in $T_{1\rho}^S$ values and CP rates among different ^{13}C peaks.

Recently, we proposed a new ^{13}C polarization technique in solids, named nuclear Overhauser polarization (NOP), for uniformly ^{13}C-labeled molecules with methyl groups.[3] NOP is based on the nuclear Overhauser effect (NOE) and a ^{13}C−^{13}C recoupling scheme of ^{13}C−^{1}H dipolar-assisted rotational resonance (DARR).[4,5] In DARR, the ^{13}C−^{1}H dipolar interaction is recovered by the ^{1}H radio frequency (rf) irradiation with the intensity ν_1 satisfying the rotary-resonance condition $\nu_1 = n\nu_R$ ($n = 1$ or 2),[6] where ν_R is the spinning frequency. The spectral overlap between the two relevant ^{13}C spins, which is required for efficient polarization transfer through rotational resonance,[7−9] is realized between a spinning side band of one ^{13}C spin and the ^{13}C−^{1}H dipolar pattern of the other ^{13}C spin and vice versa. Since we apply an rf field to ^{1}H spins in DARR, NOE enhancement would occur for ^{13}C spins in fast-rotating groups such as CH_3. The enhanced polarization of mobile $^{13}CH_3$ spins is then successively transferred by DARR to the other ^{13}C spins, leading to NOE enhancement of the other stationary ^{13}C spins.

Since rotational-resonance recoupling occurs between two spins with the chemical shift difference equal to an integral multiple of the spinning frequency, NOP may be supposed to be effective only for carboxyl, carbonyl, and aromatic carbons, whose chemical shift differences from methyl carbon chemical shifts are suitable for rotational-resonance recoupling. However, we recently found that in the second order, recoupling does occur among ^{13}C resonances with smaller chemical shift differences, such as aliphatic carbons,[5] leading to uniform signal

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(1) Hartmann, S. R.; Hahn, E. L. *Phys. Rev.* **1962**, *128*, 2042−2053.
(2) Pines, A.; Gibby, M. G.; Waugh, J. S. *J. Chem. Phys.* **1973**, *59*, 569−590.
(3) Takegoshi K.; Terao, T. *J. Chem. Phys.* **2002**, *117*, 1700−1707.
(4) Takegoshi, K.; Nakamura, S.; Terao, T. *Chem. Phys. Lett.* **2001**, *344*, 631−637.
(5) Takegoshi, K.; Nakamura, S.; Terao, T. *J. Chem. Phys.* **2003**, *118*, 2325−2341.
(6) Oas, T. G.; Griffin, R. G.; Levitt, M. H. *J. Chem. Phys.* **1988**, *89*, 692−695.
(7) Andrew, E. R.; Clough, S.; Farnell, L. F.; Gledhill, T. D.; Roberts, I. *Phys. Lett.* **1966**, *21*, 505−506.
(8) Raleigh, D. P.; Levitt, M. H.; Griffin, R. G. *Chem. Phys. Lett.* **1988**, *146*, 71−76.
(9) Colombo, M. G.; Meier, B. H.; Ernst, R. R. *Chem. Phys. Lett.* **1988**, *146*, 189−196.

Table 1. Signal Enhancement Factors and Area Intensity Rates in Lyophilized [U-^{13}C,^{15}N]-Yhh[a]

	enhancement factor					area intensity (%)						
method, τ_{CP}/τ_{NOP}	CP, 1 ms	CP, 4 ms	NOP, 4 s	NOP, 8 s	NOP, ∞	90° pulse	CP, 1 ms	CP, 4 ms	NOP, 4 s	NOP, 8 s	content (%)	NOP rate (s^{-1})
carbonyl	0.23	0.38	1.55	1.85	1.93	23.5	7.7	16.4	23.4	23.8	24.2	0.39
aromatic	0.55	0.44	1.40	1.68	1.81	12.2	9.7	9.6	10.9	11.2	10.2	0.35
aliphatic	0.88	0.63	1.59	1.84	1.82	64.3	82.7	74.0	65.7	65.0	65.7	0.53
total	0.80	0.57	1.56	1.82	1.84							0.46

[a] Best-fit parameters to the NOP−time dependence data in Figure 2a are also collated.

enhancement by NOP. It was shown that in uniformly ^{13}C-labeled samples the optimal enhancement factor in NOP is about 3.0 at the fast motion and fast transfer limit,[3] which may be larger than that in CP. Furthermore, NOP has the advantage of quantitativeness, and is very easy to carry out, being insensitive to the adjustment of rf field intensity and requiring only low rf power.[3] In this work, the usefulness of NOP in solid-state NMR studies of proteins is examined for three kinds of uniformly ^{13}C,^{15}N-labeled proteins with two different sample conditions.

Experimental Section

Samples. Recombinant uniformly ^{15}N,^{13}C-labeled Yhh ([U-^{13}C,^{15}N]-Yhh) and the [U-^{13}C,^{15}N]-RING-H2 finger domain of EL5 were expressed in *Escherichia coli* grown in M9 minimal medium with the expression vectors [pET21b(+) for Yhh and pET32a for the RING-H2 finger domain] and the host bacterial strain BL21(DE3), and purified as described previously.[10,11] Recombinant [U-^{13}C,^{15}N]thioredoxin was obtained by cleavage from the fusion protein of the RING-H2 finger domain with thioredoxin during purification of the [U-^{13}C,^{15}N]-RING-H2 finger domain protein. [U-^{13}C,^{15}N]-Yhh, the [U-^{13}C,^{15}N]-RING-H2 finger domain, and [U-^{13}C,^{15}N]thioredoxin were lyophilized at −80 °C. For the Yhh precipitated sample, solid ammonium sulfate was added to 75% saturation at 4 °C and the precipitate was recovered by centrifugation. [U-^{13}C,^{15}N]-*N*-Acetyl-L-prolylglycyl-L-phenylalanine ([U-^{13}C,^{15}N]-Pro-Gly-Phe) was synthesized by conventional Fmoc methods.

NMR Measurements. The NMR experiments were carried out on a Chemagnetics CMX-400 spectrometer operating at a resonance frequency of 100.3 MHz for ^{13}C with a CPMAS probe (Chemagnetics) having a 3.2 mm rotor system. Both the MAS frequency ν_R and the ^{1}H rf intensity ν_1 for DARR were about 20 kHz. Prior to the ^{1}H irradiation, three 90° pulses were applied to ^{13}C with an interval of 10 ms to remove ^{13}C longitudinal magnetization for correctly estimating the NOP enhancement factor. TPPM decoupling[12] was used with an optimal nutation angle and phase-modulation angle of 180° and ±15°, respectively, for the rf intensity of 100 kHz and a MAS frequency of 20 kHz. For CP enhancement, the ^{1}H rf intensity was 60 kHz and the ^{13}C rf intensity was varied linearly from 75 to 85 kHz with the contact time divided into 10 segments, during each of which the ^{13}C rf intensity was kept constant. The ^{13}C and ^{1}H spin−lattice relaxation times were measured by use of Torchia's pulse sequence[13] and the inversion recovery method, respectively. Both ^{13}C and ^{1}H spin−lattice relaxation times in the rotating frame were measured with an rf field intensity of 57 kHz.

Results and Discussion

Figure 1 shows ^{13}C MAS spectra of lyophilized [U-^{13}C,^{15}N]-Yhh by a single 90° pulse without NOP (a), by CP (b, c), and by a 90° pulse with NOP (d, e). The repetition time of 50 s for the single 90° pulse experiment (Figure 1a) is much longer than the longest ^{13}C T_1 value (∼1.26 s), so that the spectrum ensures the full signal intensities at thermal equilibrium. In the CP experiments, a relaxation interval of 4 s (^{1}H $T_1 \sim 0.9$ s) and a CP contact time (τ_{CP}) of 1 ms (b) or 4 ms (c) were used. In the NOP experiments, the NOP time (τ_{NOP}) was 4 s (d) or 8 s (e) with the DARR condition of $\nu_1 = \nu_R = 20$ kHz. In Table 1, the area intensities of the carbonyl, aromatic, and aliphatic carbon signals in Figure 1b−e are listed in values relative to the corresponding intensity in Figure 1a, together with the CP and NOP enhancement factors of the individual and total signals.

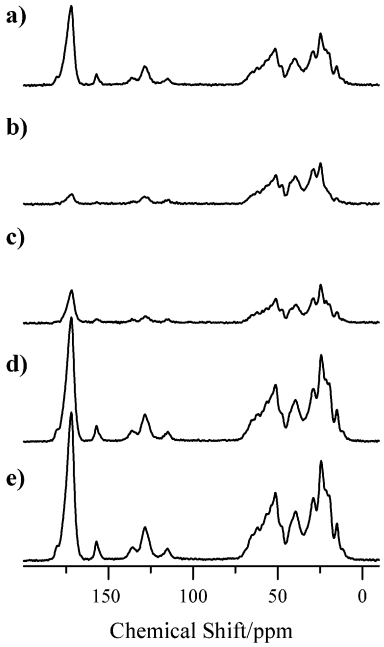

Figure 1. ^{13}C MAS spectra of lyophilized [U-^{13}C,^{15}N]-Yhh. (a) Observed by a ^{13}C 90° pulse without NOP. The relaxation interval was 50 s, much longer than the ^{13}C spin−lattice relaxation time of 1.26 s. (b, c) Obtained by CP with contact times of 1 and 4 ms, respectively. The relaxation interval was 4 s. (d, e) Obtained by NOP with times (τ_{NOP}) of 4 and 8 s, respectively. Thirty-two FIDs were accumulated for each experiment, and the spectra are plotted on the same amplitude scale so that they can be directly compared.

Figure 2 shows τ_{NOP} (a) and τ_{CP} dependence (b) of the area intensities of the carbonyl, aromatic, and aliphatic ^{13}C signals in lyophilized [U-^{13}C,^{15}N]-Yhh. Each signal intensity is normalized by the corresponding area intensity observed for a ^{13}C 90° pulse with a relaxation interval of 50 s. The observed τ_{NOP} dependence (a) is fitted to the following single-exponential function:

$$S(t) = \eta_{NOP}\{1 - \exp(-k_{NOP}t)\} \quad (1)$$

with the NOP enhancement factor at an infinite time (η_{NOP}) and the apparent NOP buildup rate k_{NOP} as fitting parameters.

(10) Ishii, Y.; Yamada, H.; Katoh, E.; Yamazaki, T.; Shindo, H.; Mizuno, T. *Microbiol. Biosci. Biotechnol. Biochem.* **2000**, *64*, 799−807.
(11) Katoh, S.; Hon, C.; Tsunoda, Y.; Murata, K.; Takai, R.; Minami, E.; Yamazaki, T.; Katoh, E. *J. Biol. Chem.* **2003**, *270*, 1269−1276.
(12) Bennett, A. E.; Rienstra, C. M.; Auger, M.; Lakshmi, K. V.; Griffin, R. G. *J. Chem. Phys.* **1995**, *103*, 6951−6958.
(13) Torchia, D. A. *J. Magn. Reson.* **1978**, *30*, 613−616.

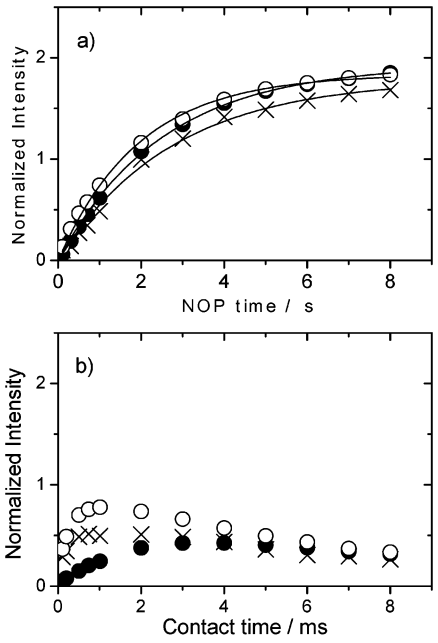

Figure 2. NOP time (a) and contact time (b) dependence of the area intensities of aliphatic (○), aromatic (×), and carbonyl (●) ^{13}C signals in lyophilized [U-^{13}C,^{15}N]-Yhh. Each signal intensity is normalized by the corresponding area intensity observed by a ^{13}C 90° pulse with a relaxation interval of 30 s. The solid lines in panel a are the best-fit curves to eq 1 with the best-fit parameters collated in Table 1.

The solid curves in Figure 2a are the best-fit curves with parameters listed in Table 1. Figures 1 and 2 and Table 1 clearly show the following results: the spectral intensity is weaker in the CP spectra than in the 90° pulse spectra ($\eta_{CP} \sim 0.2-0.9$), while in NOP it is substantially stronger ($\eta_{NOP} \sim 1.4-1.9$). Particularly, the signal intensity of the carbonyl carbon is exceedingly low in CP but much higher in NOP. This is ascribed to the fact that CP relies on the ^{13}C−^{1}H dipolar couplings, while NOP utilizes the ^{13}C−^{13}C dipolar couplings. Moreover, the signal intensities are far from quantitative in CP but remarkably quantitative in NOP, as shown in Table 1. The area−intensity rates obtained by a 90° pulse for fully relaxed ^{13}C spins naturally agree well with their carbon contents, while in CP spectra observed at both $\tau_{CP} = 1$ ms (the whole intensity maximum) and 4 ms (the carbonyl carbon intensity maximum), the rates exceedingly deviate from the contents. On the other hand, NOP presents very good agreements between the area intensity rates and the contents for both repetition times, the differences being below 1%.

In the above, we compared the enhancement factors per scan. It should be pointed out that, even for a per-time basis, CP does not have an appreciable advantage over the single pulse excitation because ^{13}C T_1 of a uniformly ^{13}C-labeled protein is comparable to ^{1}H T_1. In the following, we compare the sensitivity obtained in unit time by single 90° pulse experiments (NOP) with that by CP experiments. The improvement of the signal-to-noise ratio by signal accumulation in unit time depends on a repetition time t_r as

$$\eta(t_r) = \{1 - \exp(-kt_r)\}/\sqrt{t_r} \qquad (2)$$

where k is the relaxation rate. Equation 2 leads the optimum sensitivity at $t_r \sim 1.26k^{-1}$.[14] In the NOP case, the relevant relaxation rate is the NOP rate (k_{NOP}), which is ca. 0.5 s^{-1} for the three samples. For example, the best-fit k_{NOP} values of lyophilized [U-^{13}C,^{15}N]-Yhh in Table 1 are 0.35∼0.53 s^{-1}. Hence, the optimal repetition time is ca. 2.5 s. On the other hand, for the CP experiment, the relevant relaxation rate is the ^{1}H spin−lattice relaxation rate $T_1^{-1} \sim 1.1$ s^{-1} for lyophilized [U-^{13}C,^{15}N]-Yhh. Hence, the optimal repetition time is 1.13 s. Hence, the gain of the sensitivity enhancement factor in unit time by replacing CP with NOP is given by $(\eta_{NOP}/\eta_{CP})\sqrt{1.1/2.5} \sim 1.5$: even considering the sensitivity on a per-time basis, NOP is still advantageous compared to CP. Last, we would like to point out that, for a 1D NOP experiment, the 90° flip angle does not constitute the best choice and pulsing rapidly with a small pulse flip angle should further improve the sensitivity.[15] We calculated the gain numerically for the NOP experiment; however, the gain in the enhancement factor with a pulse angle of 10° is not significant (∼1.15).

The τ_{CP} dependences of aromatic and aliphatic carbon signals in Figure 2b show almost the same behavior and their signal intensities reach maximum at τ_{CP} of 1 ms, while the carbonyl carbon signal is maximized at about 4 ms. This large dissimilarity between the two types of signals obtained by CP is attributed to the differences of the CP buildup rate and the ^{13}C spin−lattice relaxation time in the rotating frame ($T_{1\rho}^{C}$) between the two types. We obtained the apparent CP buildup rate ($1/T_{IS}$) from the initial slope of the τ_{CP} dependence; the observed T_{IS} and $T_{1\rho}^{C}$ values are 1.0 and 2.1 ms, respectively, for the aromatic carbon signals, 0.7 and 1.9 ms, respectively, for the aliphatic carbon signals, and 4.2 and 11.0 ms, respectively, for the carbonyl carbon signals. The ^{1}H spin−lattice relaxation time in the rotating frame ($T_{1\rho}^{H}$) is ca. 2.4 ms for all carbon signals. The buildup time constants of the aliphatic and aromatic carbon signals are about 1 ms, while the signals decay with the short $T_{1\rho}^{C}$ and $T_{1\rho}^{H}$ relaxation times of about 2 ms. Hence, the signal reaches maximum at ca. 1 ms and the intensity is far less than the theoretical maximum obtainable without relaxation. For the carbonyl carbon signal, the buildup is very slow ($T_{IS} \sim 4$ ms). Hence, a longer contact time is required to obtain higher enhancement. However, the maximum intensity obtained at 4 ms is still significantly low because of the loss of ^{1}H polarization due to short $T_{1\rho}^{H}$.

It is noted that the observed T_{IS} values of lyophilized [U-^{13}C,^{15}N]-Yhh ($T_{IS} = 1.0$, 0.7, and 4.2 ms, for the aromatic, aliphatic, and carbonyl carbons, respectively) are much longer than those for rigid molecules. For comparison, we measured T_{IS} of [U-^{13}C,^{15}N]-Pro-Gly-Phe (not shown) to be 50, 36, and 350 μs, for the aromatic, aliphatic, and carbonyl carbon signals, respectively. The long T_{IS} values and the short $T_{1\rho}^{C}$ and $T_{1\rho}^{H}$ values observed in lyophilized [U-^{13}C,^{15}N]-Yhh indicate that protein molecules are significantly mobile with the characteristic motional frequency comparable to the rf intensity (∼70 kHz) even in lyophilized samples. This results in the observed low efficiency of CP enhancement.

Here, let us examine effects of molecular motion on NOP enhancement. For a spin system consisting of ^{1}H spins (I) and N ^{13}C spins (L) coupled to one ^{13}C spin of a rotating CH_3 group (S), the average NOP enhancement factor for the L spins is given under the steady-state condition with ^{1}H saturation as[3]

(14) Ernst, R. R.; Morgan, R. E. *Mol. Phys.* **1973**, *26*, 49−74.
(15) Ernst, R. R.; Anderson, W. A. *Rev. Sci. Instrum.* **1966**, *37*, 93−100.

$$\eta_{NOP} = 1 + \frac{kT_1^L}{1 + k(T_1^L + NT_1^S)}\xi_{NOE} \quad (3)$$

with

$$\xi_{NOE} = \frac{T_1^S}{T_1^{SI}}\frac{I_0}{S_0} \quad (4)$$

where k is the average polarization transfer rate between the S and L spins, T_1^X is the spin−lattice relaxation time of the X spin ($X = S$ or L), T_1^{SI} is the cross relaxation time between the I and S spins, and X_0 denotes the thermal-equilibrium magnetization of the X spin. At the extreme narrowing condition, where the rotational correlation time of the methyl group is much shorter than the Larmor precession period, we have the maximum ξ_{NOE} of

$$\xi_{NOE}^{max} = \frac{1}{2}\frac{I_0}{S_0} \quad (5)$$

Furthermore, eq 3 indicates that when both conditions of the fast $^{13}C-^{13}C$ polarization transfer limit ($kT_1^L \gg 1$) and the long spin−lattice relaxation limit of the L spins ($T_1^L \gg NT_1^S$) are fulfilled, the NOP enhancement factor η_{NOP} reaches a maximum of $1 + \xi_{NOE}$. Hence, the maximum NOP enhancement of $\eta_{NOP} = 2.99$ is attained when the three conditions of extreme narrowing, fast transfer limit, and long L relaxation are fulfilled simultaneously. However, the observed enhancement factors $\eta_{NOP} \sim 1.8$ are significantly smaller than the maximum factor of 2.99. For proteins, the extreme narrowing condition is likely to be satisfied. In fact, in *Streptomyces* subtilisin inhibitor (SSI), the correlation time of the methyl rotation is estimated to be 9.6×10^{-12} s at 300 K from the ^1H T_1 data.[16] Similar short correlation times have been found also for ribonuclease A, α-chymotrypsin, and lysozyme.[17] On the other hand, it is not apparent whether the other two conditions for the maximum NOP enhancement are realized or not.

To quantitatively examine the long relaxation condition ($T_1^L \gg NT_1^S$), we attempted to measure ^{13}C T_1 values of lyophilized [U-^{13}C,^{15}N]-Yhh by a conventional T_1 experiment; however, except for the aliphatic carbons showing single-exponential behavior with $T_1 \sim 1.26$ s, the relaxation processes of the other two carbon groups are influenced significantly by the $^{13}C-^{13}C$ spin diffusion. Hence, it was difficult to determine ^{13}C T_1 for the aromatic and carbonyl carbons by the conventional T_1 experiment. The T_1 of methyl groups should be shorter than the T_1 value of aliphatic carbons. Suppose the T_1 value of methyl carbons of a protein is ∼0.1 s: the long relaxation time condition for the nonmethyl carbons requires T_1^L much longer than 1 s with $N = 10$. It appears that the long relaxation time condition can be satisfied for rigid carbons; for example, the observed ^{13}C T_1 values of L-alanine are 0.038, 4.0, and 13 s for the methyl, methine, and carbonyl carbons.[18] However, it should be noted that the extreme narrowing condition for methyl rotation leads to longer ^{13}C T_1^S, making the condition more difficult to satisfy.

As for the fast $^{13}C-^{13}C$ polarization transfer limit ($kT_1^L \gg 1$), the motion that lowers the CP enhancement would retard the $^{13}C-^{13}C$ polarization transfer by motional averaging of $^{13}C-^{13}C$ dipolar interactions. With these in mind, the observed small enhancement factors $\eta_{NOP} \sim 1.8$ can roughly be explained by putting $T_1^L \sim 10$ s, $T_1^S \sim 1$ s, $k \sim 1$ s^{-1}, and $N = 10$. Thus, molecular motion with its characteristic frequency comparable to the rf intensity (∼70 kHz) affects both CP and NOP. For CP, motional averaging of $^{13}C-^1H$ and $^1H-^1H$ dipolar interactions causes short $T_{1\rho}^{I,S}$ and long T_{IS} values, leading to the observed small CP enhancement factors. On the other hand, the motion affects NOP through averaging of the $^{13}C-^{13}C$ dipolar interactions. However, even with a reduced k, NOP enhancement can be large if the relaxation time of nonmethyl carbons is long enough to satisfy the fast $^{13}C-^{13}C$ polarization transfer limit ($kT_1^L \gg 1$). Hence, the effects of molecular motion are somewhat alleviated in NOP, when T_1^L is long.

We would like to point out that in these experiments the sample temperatures are increased ca. 30° higher than room temperature, mainly because of sample rotation. Hereafter, it may become important to perform structural studies of proteins at lower temperatures to compensate sample heating or to obtain narrower lines by use of cryoprotectants. With decreasing temperature, CP would work better because molecular motion with its characteristic frequency comparable to the rf intensity should become less appreciable. NOP efficiency would be also enhanced to some extent at some low temperatures owing to increase of the $^{13}C-^{13}C$ polarization transfer rate as exemplified by the success of NOP in rigid L-threonine.[3] However, the extreme narrowing condition for methyl rotation will not be fulfilled at sufficiently low temperatures. Hence, with decreasing temperature, the CP enhancement factor will become larger than that of NOP at a certain temperature. It should be noted that, however, even in such a situation, the superiority of NOP in quantitativeness is maintained.

Figure 3 shows τ_{NOP} (a) and τ_{CP} dependence (b) of the area intensities of the whole ^{13}C spectra in lyophilized [U-^{13}C,^{15}N]-Yhh, thioredoxin, and the RING-H2 finger domain of EL5 and Yhh precipitated from a $(NH_4)_2SO_4$-rich solution. The last one was examined because the precipitate from a $(NH_4)_2SO_4$-rich solution of protein had been suggested to improve resolution in solid-state NMR by hydration.[19] The enhancement factor by NOP in Yhh precipitation from a $(NH_4)_2SO_4$-rich solution is 1.5, being considerably smaller than that (1.8) in the lyophilized one. Molecular motion in precipitated Yhh may be more rapid than in lyophilized Yhh and reduce $^{13}C-^{13}C$ dipolar interactions to retard $^{13}C-^{13}C$ polarization transfer. Fast molecular motion in precipitated [U-^{13}C,^{15}N]-Yhh also causes the rapid decay in the τ_{CP} dependence experiment (Figure 3b). Note that for all U-^{13}C,^{15}N-labeled proteins, the optimal η_{NOP} value is about 2 times larger than the optimal η_{CP} value.

In purification processes of protein samples for solid-state NMR, a variety of reagents are usually used, such as buffer, salt, and reducing agent to prevent aggregation. Further, it was reported that some additives such as sugar[20] and $(NH_4)_2SO_4$[19] improve resolution of solid-state NMR. For these reasons, protein samples for solid-state NMR usually include a large

(16) Akasaka, K.; Takegoshi, K.; Terao, T.; Ganapathy, S. *Can. J. Chem.* **1988**, *66*, 2014−2017.
(17) Andrew, E. R.; Bryant, D. J.; Cashell, E. M. *Chem. Phys. Lett.* **1980**, *69*, 551−554.
(18) Naito, A.; Ganapathy, S.; Akasaka, K.; McDowell, C. A. *J. Magn. Reson.* **1983**, *54*, 226−235.
(19) Pauli, J.; Rossum, B.; Forster, H.; Groot, H. J. M.; Ochkinat, H. *J. Magn. Reson.* **2000**, *143*, 411−416.

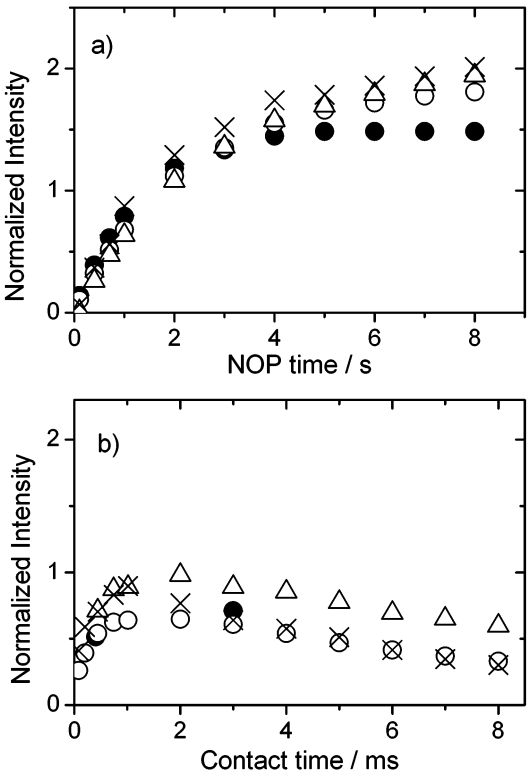

Figure 3. NOP time (a) and contact time (b) dependence of the whole area intensities of ^{13}C spectra in lyophilized [U-^{13}C,^{15}N]-Yhh (○), lyophilized [U-^{13}C,^{15}N]-thioredoxin (△), lyophilized [U-^{13}C,^{15}N]-RING-H2 finger domain of EL5 (×), and precipitate [U-^{13}C,^{15}N]-Yhh (●). Each signal intensity is normalized by the whole spectral area intensity observed by a ^{13}C 90° pulse with a relaxation interval of 30 s.

amount of impurity compounds. Figure 4 shows ^{13}C MAS spectra of the lyophilized [U-^{13}C,^{15}N]-RING-H2 finger domain containing a large amount of dithiothreitol (DTT) and Tris-HCl buffer. The CPMAS spectrum (a) of this sample shows intense signals at 57 (the methylene carbon of Tris-HCl) and 61 ppm (the quaternary carbon of Tris-HCl), but the NOP−MAS spectrum (b) does not present such peaks. This is because ^{13}C−^{13}C polarization transfer between naturally abundant ^{13}C spins is too slow to be enhanced by NOP.[3] Hence, NOP has another advantage of eliminating unwanted signals in contaminated samples.

For NOP to be effective to enhance the ^{13}C signals in a molecule, the molecule must be uniformly ^{13}C-labeled, and moreover, methyl carbons must be included with high density, and the methyl rotation frequency must be in the extremely narrowing region. The latter two conditions are not always fulfilled in general molecules. Fortunately, however, a protein has a number of methyl groups, for example, Yhh has 81 methyl

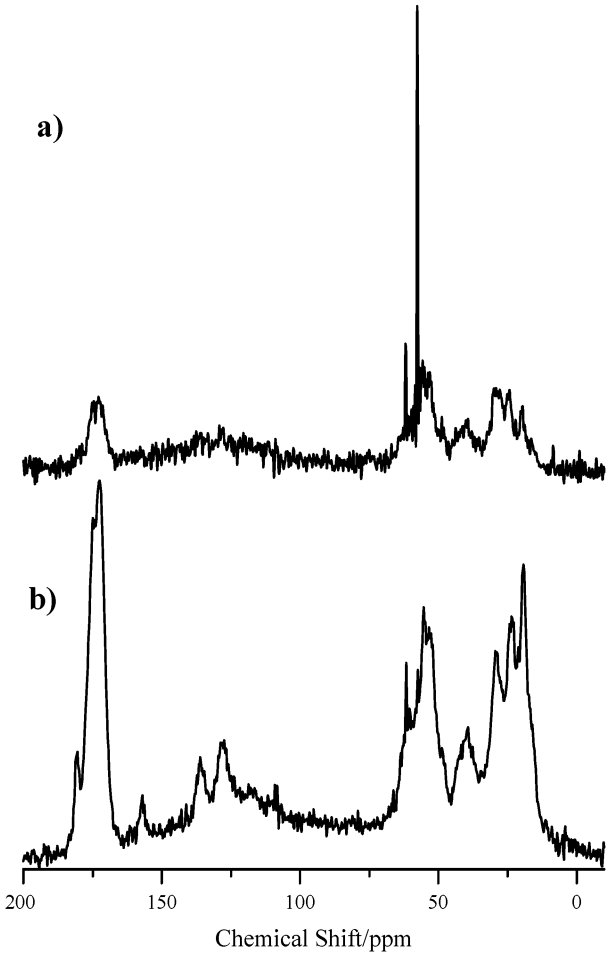

Figure 4. ^{13}C MAS spectra of the lyophilized [U-^{13}C,^{15}N]-RING-H2 finger domain of EL5 containing a large amount of DTT and buffer: (a) CP; (b) NOP.

carbons in the 401 carbons. This content of ca. 20% is enough to polarize all ^{13}C spins by NOP in several seconds. Furthermore, for a protein molecule, it is expected that molecular mobility would increase due to weaker crystallographic packing force. Hence, the correlation time of the methyl rotation in a protein is very likely much shorter than the Larmor procession period,[16,17] thus ensuring NOP enhancement. NOP would be applied for many uniformly ^{13}C-labeled proteins with the advantage of quantitativeness and larger enhancement as compared to CP with a side effect of elimination of impurity signals.

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