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Sensitivity enhanced NMR spectroscopy by quenching scalar coupling mediated relaxation: Application to the direct observation of hydrogen bonds in $^{13}C/^{15}N$-labeled proteins

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Abstract

In this paper, we demonstrate that the sensitivity of triple-resonance NMR experiments can be enhanced significantly through quenching scalar coupling mediated relaxation by using composite-pulse decoupling (CPD) or an adiabatic decoupling sequence on aliphatic, in particular alpha-carbons in $^{13}C/^{15}N$-labeled proteins. The CPD-HNCO experiment renders 50% sensitivity enhancement over the conventional CT-HNCO experiment performed on a 12 kDa FK506 binding protein, when a total of 266 ms of amide nitrogen–carbonyl carbon defocusing and refocusing periods is employed. This is a typical time period for the direct detection of hydrogen bonds in proteins via *trans*-hydrogen bond $^{3h}J_{NC'}$ couplings. The experimental data fit theoretical analysis well. The significant enhancement in sensitivity makes the experiment more applicable to larger-sized proteins without resorting to perdeuteration.

Abbreviations: 2D, 3D, two-, three-dimensional; CP, cross-polarization; CPD, composite-pulse decoupling; CT, constant-time; CSA, chemical shift anisotropy; DD, dipole–dipole; FKBP12, 12 kDa FK506 binding protein; INEPT, insensitive nuclei enhanced polarization transfer; TROSY, transverse relaxation-optimized spectroscopy.

Introduction

Together with X-ray crystallography, nuclear magnetic resonance spectroscopy has become one of the two leading techniques for the determination of tertiary structure of macromolecules at atomic resolution. Extraordinary efforts have been made to enhance the sensitivity of triple-resonance NMR experiments, where the major handicaps remain to be the intrinsically low sensitivity and short transverse relaxation times. For protonated proteins in liquid, the major contributions to the transverse relaxation of backbone ^{13}C and ^{15}N spins originate from dipole–dipole (DD) interactions with their attached protons and chemical shift anisotropy (CSA) interactions with the external magnetic field (Abragam, 1961; Ernst et al., 1987). In contrast to ^{15}N and $^{13}C^{\alpha}$, backbone carbonyl $^{13}C'$ spins have favourable relaxation behavior at medium field strengths due to the absence of directly bound protons, and their relaxation is almost exclusively governed by interactions of their CSA tensors. As a result, HNCO (Kay et al., 1990; Grzesiek and Bax, 1992) turns out to be one of the most sensitive triple-resonance experiments. For ^{15}N-labeled polypeptide chains, the relaxation behaviour of amide ^{15}N spins has been extensively studied and well understood (Kay et al., 1989; Clore et al., 1990; Peng and Wagner, 1992; Szyperski et al., 1993). It has been found that the in-phase ($N_{x,y}$) and antiphase ($2N_{x,y}H_z$) coherences relax with different rates (Bax et al., 1990; Peng et al., 1991a, b) and the antiphase coherence relaxes faster because it is affected by DD interactions with remote protons. On the other hand, the two components of

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antiphase coherence also relax with different rates, in contrast to the in-phase coherence which relaxes with an average rate constant, due to the 'constructive' and 'destructive' cross-correlation between DD and CSA interactions (Goldman, 1984; Boyd et al., 1990; Brüschweiler and Ernst, 1992; Kay et al. 1992; Palmer et al., 1992) and larger molecules at higher fields display bigger differences. This phenomenon has been successfully exploited in the TROSY experiment (Pervushin et al., 1987) to enhance the sensitivity of triple-resonance NMR experiments for macromolecules, especially in deuterated forms at high fields (Salzmann et al., 1998; Yang and Kay, 1999). However, for non-deuterated $^{13}C/^{15}N$-labeled proteins, the difference in the amide ^{15}N transverse relaxation rates between the in-phase and antiphase coherence with respect to $^{13}C^{\alpha}$ spins has been largely neglected in the conventional triple-resonance experiments (Bax and Grzesiek, 1993) because the duration of INEPT (Morris and Freeman, 1979) magnetization transfer steps involving transverse ^{15}N coherence is usually not very long.

In this work, we demonstrate that the effects of DD interactions between aliphatic carbon $^{13}C^{ali}$, in particular the backbone $^{13}C^{\alpha}$ spins, and their attached protons on the transverse relaxation rates of backbone amide ^{15}N spins may be significant, especially when a long magnetization transfer period is required for the observation of long-range connectivities, such as in HNCO-type experiments for observation of hydrogen bonds in proteins (Cordier and Grzesiek, 1999; Cornilescu et al., 1999a, b; Wang et al., 1999). We also demonstrate that this contribution can be suppressed easily by employing appropriate composite or adiabatic pulse heteronuclear band-selective decoupling sequences, thereby enhancing the sensitivity of these NMR experiments.

Theory

To illustrate the effect of $^{13}C^{\alpha}$-$^{1}H^{\alpha}$ DD interaction on the amide ^{15}N transverse relaxation, consider the scalar coupled spin-1/2 system, $-^{13}C^{\alpha}(^{1}H^{\alpha})-^{13}C'-^{15}N(^{1}H^{N})-^{13}C^{\alpha}(^{1}H^{\alpha})-$, of a dipeptide segment of a globular protein backbone. During the magnetization transfer periods between backbone amide ^{15}N and carbonyl $^{13}C'$ in the regular constant-time (CT) HNCO experiment (Grzesiek and Bax, 1992), α-carbon spins are usually decoupled by $^{13}C'$ selective 180° pulses in the middle of these defocusing/refocusing periods, so that the ^{15}N transverse relaxation is modulated by the oscillation among in-phase and antiphase magnetization terms N_x, $2N_yC_z^i$, $2N_yC_z^s$, and $4N_xC_z^iC_z^s$, where $C_z^{i/s}$ represent intraresidual and sequential α-carbon magnetizations. When the total ^{15}N–$^{13}C'$ defocusing and refocusing periods, $4 \times T_{NC'}$, in the CT-HNCO experiment are sufficiently long, namely $2 \times T_{NC'} \gg 1/^{n}J_{NC\alpha}$, the condition $(2\pi^{n}J_{IS})^2 \gg (R_N^{anti} - R(N_x))^2$ holds for $^{1}J_{NC\alpha}$ and $^{2}J_{NC\alpha}$, where R_N^{anti} stands for the transverse relaxation rates of the anti-phase coherence. As a result, ignoring cross-correlation and cross-relaxation effects, the ^{15}N relaxation rate is an average: $R_N^{av} = 1/4\{R(N_x) + R(2N_yC_z^i) + R(2N_yC_z^s) + R(4N_xC_z^iC_z^s)\}$ (Cavanagh et al., 1996) if the DD and CSA contributions from the directly coupled carbonyl carbon are not considered. On the other hand, provided that the aliphatic carbons, in particular the α-carbons, are efficiently decoupled by a composite-pulse decoupling (CPD) or an adiabatic decoupling sequence, the transverse relaxation rate of ^{15}N in the CPD-HNCO experiment is that of the in-phase coherence alone. When $2 \times T_{NC'}$ is long, as in the long-range HNCO experiments, to a good approximation, the difference in the relaxation rates of CT-HNCO and CPD-HNCO experiments is therefore (Abragam, 1961; Wagner, 1993):

$$\Delta R_N^{av} \cong R_N^{av} - R(N_x)$$
$$\cong d_{HC}\{3\boldsymbol{J}(\omega_C) + \boldsymbol{J}(\omega_H - \omega_C) + 6\boldsymbol{J}(\omega_H + \omega_C)\} \quad (1)$$

where d_{HC} is the dipolar coupling constant between the proton and its attached carbon-13. $\boldsymbol{J}(\omega) = (2/5)\{S^2\tau_c/[1 + (\tau_c\omega)^2] + (1 - S^2)\tau/[1 + (\tau\omega)^2]\}$ represents the spectral density function at frequencies ω, in which τ_c (in ns) is the overall rotational correlation time and S^2 is the generalized order parameter from the model-free formalism for an isotropically tumbling protein (Lipari and Szabo,1982a, b). $1/\tau = 1/\tau_c + 1/\tau_e$, where τ_e (in ps) is the effective correlation time for characterization of the faster local motion. Since the CSA of aliphatic carbons and the DD interactions between ^{15}N and $^{13}C^{\alpha}$ are small, they have been ignored from Equation 1, thus the whole value is dominated by the terms of DD interactions between α-carbons and their attached protons.

The result of Equation 1 bears several important consequences. First of all, the positive value of this difference indicates that ^{15}N magnetization in the CPD-HNCO experiment relaxes more slowly than in its counterpart, CT-HNCO. In other words, the sensitivity of HNCO-type experiments can be enhanced significantly by employing CPD on aliphatic carbons.

Secondly, the sensitivity enhancement is tremendous for small biomolecules with short overall correlation times because the spectral density functions in Equation 1 are sampled at higher frequencies. Indeed, this point has been demonstrated for a set of novel triple-resonance experiments used for the assignment of the flexible 'tail' in the full-length human prion protein (Liu, 1999; Liu et al., 2000). However, the enhancement may also be significant for medium-sized proteins.

Figure 1a is the correlation plot of backbone ^{15}N transverse relaxation rates versus the overall correlation time τ_c of a globular protein at 14.1 T (600 MHz for ^{1}H) magnetic field. The curve *a* is for the in-phase coherence $R(N_x)$ when $^{13}C^{ali}$ spins are decoupled with CPD as in the CPD-HNCO experiment. The curve *b* is for $R_N^{av} = R(N_x) + \Delta R_N^{av}$, where ΔR_N^{av} is expressed in Equation 1 as in the CT-HNCO experiment. Figure 1b shows the sensitivity enhancement of the CPD-HNCO experiment over the CT-HNCO experiment versus the ^{15}N-^{13}C′ defocusing and refocusing periods, $4 \times T_{NC'}$, for globular proteins with different overall correlation times.

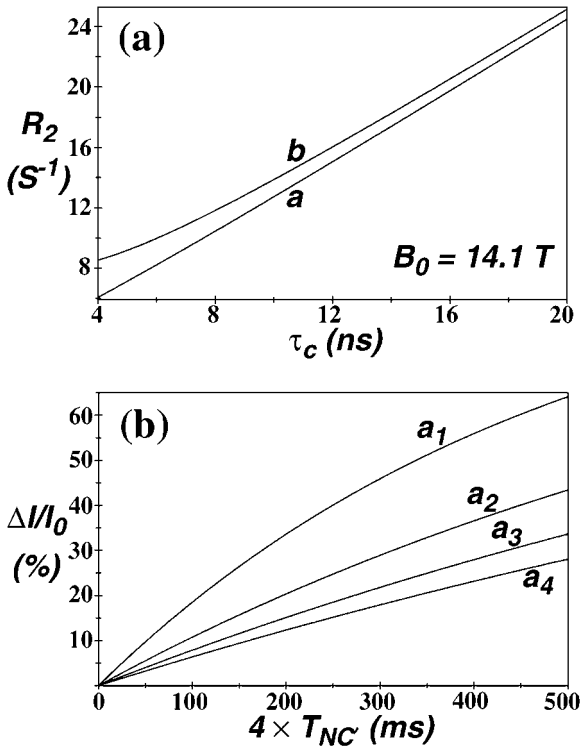

Figure 1. (a) Prediction of protein backbone ^{15}N transverse relaxation rates (R_2) versus the overall correlation time (τ_c). The curve *a* is for the in-phase coherence calculated using $R(N_x)$ (Abragam, 1961) and the curve *b* results from the averaging on in-phase and antiphase coherence, using $R_N^{av} = R(N_x) + \Delta R_N^{av}$ of Equation 1. (b) Correlation of the sensitivity enhancement of CPD-HNCO over CT-HNCO with the total ^{15}N-^{13}C′ defocusing/refocusing period $4 \times T_{NC'}$ for globular proteins with different average overall correlation times (τ_c). Curves a_1–a_4 were obtained with τ_c = 5.0, 10.0, 15.0, 20.0 ns, respectively. An average order parameter $S^2 = 0.88$ and effective correlation time $\tau_e = 50$ ps were used in the calculation for spectral densities. Diatomic distances between proton and its attached nitrogen-15 or carbon-13 are $r_{HN} = 1.02$ Å, $r_{HC} = 1.09$ Å, respectively. The CSA of backbone ^{15}N was set to −160 ppm (Hiyama et al., 1988; Tjandra et al., 1996). The intraresidual $^2J_{NC\beta}$ and $^3J_{NC\gamma}$ couplings and sequential $^3J_{NC\beta}$ couplings may be as large as 1.0 Hz, 2.5 Hz, and 0.5 Hz, respectively (Bystrov, 1976; Hu and Bax, 1997a, b; Konrat et al., 1997). Depending on the setting of ^{15}N–^{13}C defocusing and refocusing periods, $4 \times T_{NC'}$, a certain extent of ^{15}N-$^{13}C^{\beta}$ and ^{15}N–$^{13}C^{\gamma}$ antiphase magnetization will build up during these delays if either residue *i* or its N-terminal sequential residue, $i - 1$, is a $^{13}C^{\beta}H_2$-containing or $^{13}C^{\gamma}H_2$-containing residue. Then, the $^{13}C^{\beta/\gamma}$–$^1H^{\beta/\gamma}$ DD interactions will also contribute to the backbone ^{15}N transverse relaxation and in particular those from $^{13}C^{\gamma}$ should not be neglected.

Experimental and results

To evaluate the effect of scalar coupling mediated relaxation in proteins, a sensitivity comparison of the CT-HNCO (Grzesiek and Bax, 1992) and CPD-HNCO experiments has been made. In the regular CT-HNCO experiment the total ^{15}N-^{13}C′ defocusing/refocusing period is about 50 ms and the sensitivity enhancement by using CPD on aliphatic carbons is not tremendous (see also Figure 1b). However, when it is required to lengthen these periods for observing long-range connectivities, such as hydrogen bonds in proteins (Cordier and Grzesiek, 1999; Cornilescu et al., 1999a, b; Wang et al., 1999), where the whole ^{15}N-^{13}C′ defocusing/refocusing period is usually $4 \times T_{NC'} \cong$ 266 ms, which is sufficiently long to create antiphase coherence, the enhancement becomes remarkable.

The experiment is demonstrated on a 12 kDa (107 amino acid residues) FK506 binding protein, FKBP12 (Harding et al., 1989; Sielierka et al., 1989; Rosen et al., 1990; Michnick et al., 1991; Van Duyne et al., 1991). The expression and purification of this protein have been described previously (Standaert et al., 1990). The NMR sample contains 3.1 mM ^{13}C/^{15}N-labeled protein dissolved in 250 μL of 93%/7% H_2O/D_2O with 25 mM sodium acetate-d_3

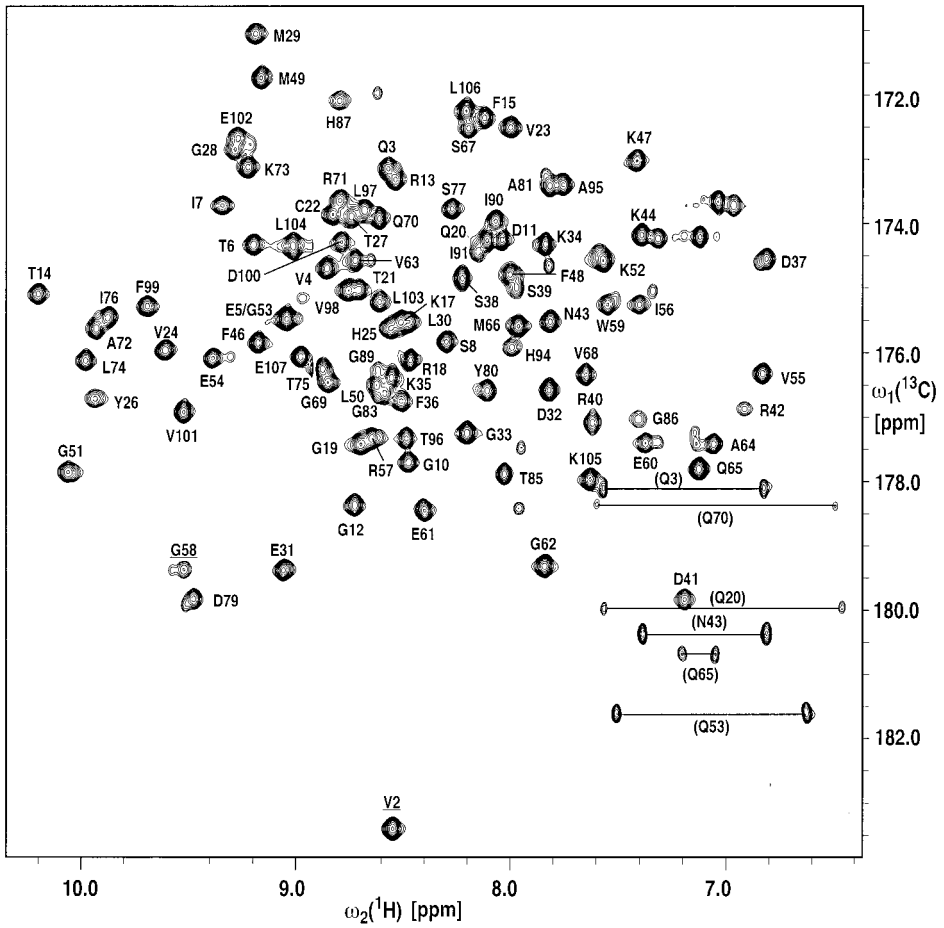

Figure 2. 2D H(N)CO spectrum of FKBP12 with 4 × 12.0 ms ^{15}N-^{13}C′ defocusing and refocusing periods using CPD on α-carbons. The time-domain data matrix consisted of 100* (t_1) × 736* (t_2) complex points with acquisition times of 45.5 ms (t_1) and 73.6 ms (t_2), respectively. Data were collected with 4 scans and resulted in a measuring time of 0.5 h. The assignment is represented with one-letter code followed by the residue number. The folded V2 and G58 peaks are underlined. Signals from each Asn and Gln residue side chain are linked with horizontal lines.

at pH 5.0. NMR spectra were collected at 25 °C on a Varian Inova 600 MHz (^{1}H) instrument equipped with a z-axis pulsed field gradient probehead. Carbonyl carbon assignments (see Figure 2) were established by correlating them with the already assigned amide chemical shifts (Rosen et al., 1991; Xu et al., 1993) using the 3D CPD-HNCO experiment. Over 20 cross peaks involving hydrogen bonds have been observed and assigned through the combined use of 3D CPD-HNCO and 2D CPD-H(N)CO data. Figure 3 shows a comparison of 2D H(N)CO spectra, (a) with and (b) without employing CPD on aliphatic carbons for a total period of 4 × 66.5 = 266 ms. Clearly, the peak intensity in the spectrum with CPD is much higher than that without using CPD, and a range of 30%–60% (average: 50%) enhancement is achieved. The enhancement goes up to over 100% when the CPD period is set to 4 × 100.0 ms (data not shown). Some peaks that are observed in Figure 3a are too weak to be seen in Figure 3b, indicating the importance of using the sensitivity-enhanced experiment for observing weak signals that are generated from small *trans*-hydrogen bond $^{3h}J_{NC'}$ couplings.

Discussion

The backbone dynamics of the protein FKBP12 and the FKBP12/FK506 complex has been studied using ^{15}N relaxation data by Moore and co-workers (Cheng et al., 1993, 1994). The final optimized overall cor-

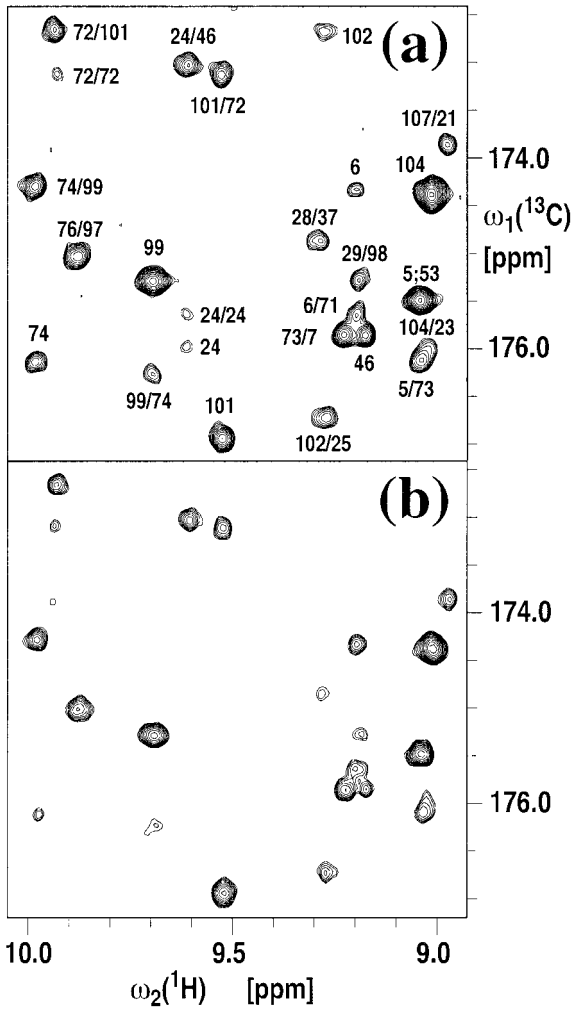

Figure 3. A comparison of a selected region of the 2D H(N)CO spectra of $^{13}C/^{15}N$-labeled FKBP12 (a) with and (b) without employing composite or adiabatic pulse decoupling on backbone aliphatic carbons during the ^{15}N-$^{13}C'$ defocusing/refocusing periods. Aliphatic carbon $^{13}C^{ali}$ band-selective decoupling was achieved with the adiabatic decoupling sequence WURST-20 (Kupce and Freeman, 1995) centered at 43 ppm, using 2 ms pulse length, 20 kHz sweep-width, 1.96 kHz amplitude, and applying a five-step super phase cycle (Tycko, 1985) to reduce decoupling side-bands. Proton decoupling using the DIPSI-3 sequence (Shaka et al., 1988; Cavanagh and Rance, 1992) with a 3.55 kHz field strength was applied during most parts of the pulse sequences. The total ^{15}N-$^{13}C'$ defocusing/refocusing delay was set to 4×66.5 ms. Both spectra resulted from the time-domain data matrix consisting of $100^*(t_1) \times 736^*(t_2)$ complex data points with acquisition times of 45.5 ms (t_1) and 73.6 ms (t_2), respectively. The entire measuring time was 19 h with 200 scans for each spectrum. Cross peaks are marked with *a/b*, where *a* is the residue number of the amide resonance and *b* the residue number corresponding to the *J*-coupled carbonyl. For one-bond $^1J_{NC'}$ connectivities to the preceding residue, only the amide is numbered.

relation time (τ_c) was about 9.0 ns and the average order parameter (S^2) was 0.88 at 500.13 MHz (^1H) and 30 °C. The predicted sensitivity enhancement of the CPD-HNCO experiment over the CT-HNCO experiment from the simulation using these parameters is 28% for $4 \times T_{NC'} = 266$ ms (see Figure 1b). However, if taking into account all the $^{13}C^{\beta/\gamma}$–$^1H^{\beta/\gamma}$ DD interactions for $^{13}C^{\gamma}H_2$-containing residues, the enhancement should be higher and a good agreement with the experimental results is demonstrated. For even longer $T_{NC'}$ periods the smaller *J*-coupling mediated $^{13}C^{\beta/\gamma}$-$^1H^{\beta/\gamma}$ DD interactions play a more significant role, in particular for $^{13}C^{\gamma}H_2$-containing residues. Indeed, the sensitivity enhancement of CPD-HNCO over CT-HNCO goes up to 100% when setting $4 \times T_{NC'} = 4 \times 100.0 = 400.0$ ms (data not shown), which is two times higher than the result of prediction (see Figure 1b). The observed enhancement shows a quite large dispersion, ranging from 30% to 60% (Figure 3). This probably reflects the residue and side chain conformation dependent features of the *J*-coupling mediated relaxation. Finally, it is very important to consider the difference in dynamics between the backbone and side chains of proteins. Because generally side chains have a higher flexibility than the backbone, the average order parameter (S^2) obtained from the backbone relaxation data is larger than the *effective* order parameter for side chains. Consequently, a larger sensitivity enhancement can be expected in the theoretical simulation if a smaller order parameter and a larger effective correlation time, τ_e, are used. Again, the local dynamics of side chains is also residue and conformation dependent. Moreover, as indicated from the prediction (Figure 1b), a much higher sensitivity enhancement can be achieved for smaller proteins, such as ubiquitin ($\tau_c \sim 5.2$ ns).

The same principle is also applicable to the HNCA-type experiments performed on either deuterated or non-deuterated proteins. During the ^{15}N-$^{13}C^{\alpha}$ defocusing/refocusing period, the carbonyl $^{13}C'$ must be efficiently decoupled with CPD, otherwise the build-up of ^{15}N antiphase (with respect to $^{13}C'$) coherence will introduce the $^{13}C'$ CSA into the ^{15}N transverse relaxation rate, decreasing the experimental sensitivity, in particular at high magnetic field. However, such *J*-coupling mediated CSA relaxations are generally small because the relevant spectral densities are sampled at high frequencies.

Observation and analysis on faster relaxation of the antiphase coherence as compared to in-phase coherence have been reported (Vold and Vold, 1976; Bax

et al., 1990; London, 1990; Peng et al., 1991a, b; Harbison, 1993) and are commonly referred to as 'scalar relaxation' or 'scalar relaxation of the second kind' (Bax et al., 1990; London, 1990) following Abragam's (1961) nomenclature. The ^{1}H-^{1}H dipolar broadening of multiple-quantum coherence was thoroughly discussed by Bax and co-workers in a comparison of different modes of two-dimensional reverse-correlation NMR experiments for the study of proteins (Bax et al., 1990). For large biomolecules in solution, the ^{1}H-^{1}H dipolar cross-relaxation is very efficient; as a result, the proton longitudinal relaxation time is short and renders a typical example of scalar relaxation of the second kind. On the other hand, there was also concern about the validity of the theoretical expression of the scalar relaxation of the second kind when the longitudinal relaxation time of the scalar coupled partner is not short compared to the *J*-coupling constant (Peng, 1991a). The observation in this study is exactly the case. As shown in Equation 1, the contribution of $^{13}C^{\alpha}$-$^1H^{\alpha}$ DD interaction to the transverse relaxation of backbone ^{15}N spins is more efficient for smaller proteins with shorter overall correlation times because the relevant spectral densities are sampled at high frequencies. For small or medium-sized proteins the longitudinal relaxation time of $^{13}C^{\alpha}$ is not short in comparison with $1/(2\pi {}^nJ_{NC\alpha}) \sim 20$ ms, where $^nJ_{NC\alpha}$ stands for backbone intraresidual or sequential $^1H^{\alpha}$-$^{13}C^{\alpha}$ scalar couplings $^1J_{NC\alpha}$ (7–11 Hz) and $^2J_{NC\alpha}$ (4–9 Hz) (Bystrov, 1976). Nevertheless, as pointed out by Bax (personal communication), scalar relaxation of the second kind is intrinsically an exchange broadening mechanism which can be either in the slow, intermediate, or fast limit. It might make more sense to refer to this sort of phenomena as 'lifetime broadening', as suggested by Bax.

Conclusions

We have shown that the *J*-coupling mediated relaxation is very important. The sensitivity of HNCO-type experiments for non-deuterated ^{13}C/^{15}N-labeled proteins can be enhanced tremendously by employing CPD on aliphatic carbons, in particular when a long ^{15}N-$^{13}C'$ defocusing/refocusing period is required. With the CPD sensitivity-enhanced experiment the size of macromolecules used for observing hydrogen bonds can be increased possibly up to 15 kDa without resorting to deuterated materials. For deuterated proteins a high gain in sensitivity might not be expected because the corresponding strong ^{1}H-^{13}C DD relaxation is absent and the experiment can be optimized with TROSY (Pervushin et al., 1997). However, employing CPD on aliphatic carbons is also useful for quenching the second order deuteron quadrupolar relaxation.

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