Use of $^{13}C-^{13}C$ NOE for the Assignment of NMR Lines of Larger Labeled Proteins at Larger Magnetic Fields

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The methodology for the determination of solution structures by NMR has been optimized for (labeled) biomolecules of molecular weights of 20 kDa and less.[1,2] At risk of becoming less useful for the study of molecules larger than that or molecular complexes are the triple-resonance[2] and especially HCCH[3] experiments that are at the very core of the current resonance assignment protocol. The 2D, 3D, and 4D HCCH-COSY and HCCH-TOCSY experiments that correlate the side chain resonances transfer coherence over a $^1J_{CC}$ coupling of ∼34 Hz and become insensitive when the ^{13}C line widths approach this value, as for 30 kDa proteins: one computes efficiencies of only 14% for a 13 kDa, 10% for a 20 kDa, and 8% for a 30 kDa protein.[4,5]

Here, we demonstrate that magnetization transfer using the *homonuclear dipolar interaction* (NOE) between adjacent ^{13}C nuclei should be considered to complement or replace scalar transfer in larger proteins. Figure 1A shows a calculation of the steady state $\{^{13}C\alpha\}-^{13}C\beta$ NOE difference in a multispin fragment as a function of the rotational correlation time τ_c and magnetic field B.[6] One observes that the $^{13}C-^{13}C$ NOE difference is at the onset of the large molecule regime ($(\omega_C\tau_c)^2 \gg 1$) for macromolecules with rotational correlation time τ_c of 10^{-8} s at all magnetic fields considered. In contrast to the scalar transfer, NOE efficiency improves significantly with increasing molecular size and magnetic field and is substantial in the large molecule limit, even though many relaxation mechanisms are present.[7] Although maximum $^{13}C-^{13}C$ NOEs will not be attainable[8] in the near future, sizable (−20%) NOE differences can currently be obtained at 17.6 T (750 MHz) for molecular weights around 30 kDa ($\log \tau_c = -7.65$).

Potentially more interesting is the analog of a 2D $^1H-^1H$ NOESY experiment (i.e., a $^{13}C-^{13}C$ NOESY experiment).

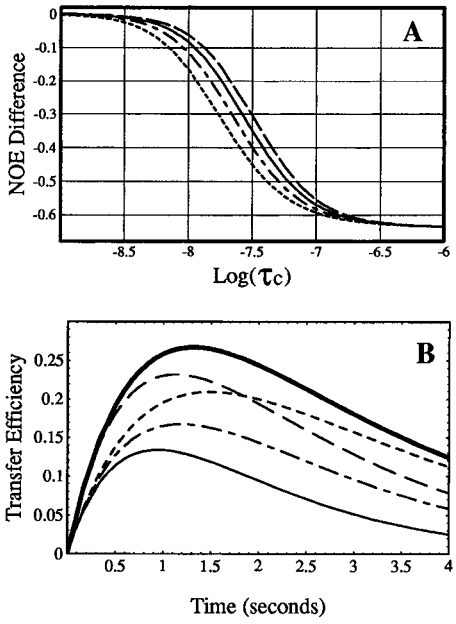

Figure 1. Computation of $^{13}C\alpha \rightarrow {}^{13}C\beta$ NOE in the molecular fragment HN, Nα, Cα, Hα, C′,Cβ, 2Hβ, Cγ, 2Hγ. (A) The steady state NOE difference for Cβ while saturating Cα. We found that this 11-spin system could be adequately described by the linear three-spin equation (see Supporting Information) $NOE^{Diff}_{\alpha\rightarrow\beta} \cong \sigma_{\beta\alpha}/(\rho_\beta - \sigma^2_{\beta\gamma}/\rho_\gamma)$, where $\sigma_{C\beta-Q} = [\mu_0/4\pi]^2(\gamma^2_{C\beta}\gamma^2_Q\hbar^2/10r^6_{C\beta-Q})\{6J(2\omega_C) - J(0)\}$ and $\rho_\beta = 2(\sigma_\perp - \sigma_{||})^2\omega^2_C/15\ \ J(\omega_C) + \sum_Q^{HN,N\alpha,C\alpha,H\alpha,C',2H\beta,C\gamma,2H\gamma}\ [\mu_0/4\pi](\gamma^2_{C\beta}\gamma^2_Q\hbar^2/10r^6_{C\beta-Q})\{3J(\omega_C) + 6J(\omega_C + \omega_Q) + J(\omega_C - \omega_Q)\}$ where μ_o is the vacuum permittivity, γ_Q is the gyromagnetic ratio of Q, $\sigma_\perp - \sigma_{||}$ is the Cβ chemical shift anisotropy, ω_c is the carbon angular Larmor frequency, r is the internuclear distance, and $\hbar$ is Planck's constant over 2π.[6] For simplicity, we assumed that $\rho_\gamma = \rho_\beta$. (B) The transient NOE (initial condition: inversion of Cα) for the $^{13}C\beta$ nucleus calculated through integration of a 3 × 3 relaxation matrix[11] for Cα, Cβ, and Cγ, where we considered 11 spins for the calculation of ρ_α and ρ_β and assumed $\rho_\gamma = \rho_\beta$. For both A and B, the Lipari−Szabo model[12] was used to model the spectral density functions using an order parameter of 0.8[7] and a local correlation time of 10^{-11} s. A value of 32 ppm was used for the Cβ CSA anisotropy.[13] Line styles in A represent, from right to left: long dashes, 11.75 T and 500 MHz ^1H; solid line, 14.09 T and 600 MHz ^1H; alternating dashes, 17.62 T and 750 MHz ^1H; short dashes, 23.5 T and 1000 MHz ^1H. Line styles in B represent: thin solid line, 20 kDa protein at 14.09 T and 600 MHz; alternating dashes, 20 kDa protein at 17.62 T and 750 MHz ^1H; short dashes, 20 kDa protein at 23.5 T and 1000 MHz ^1H; long dashes, 30 kDa protein at 17.62 T and 750 MHz ^1H; thick solid line, 30 kDa protein at 23.5 T and 1000 MHz ^1H.

Related spectra were recently demonstrated for measuring relaxation properties through $^{13}C\alpha$-^{13}CO NOE buildup.[9] Figure 1B shows computed NOE buildup curves at different conditions; it follows that the $^{13}C-^{13}C$ NOE at 1 GHz will be three times as efficient as the $^{13}C-^{13}C$ scalar transfer for a similar size molecule. For 20 kDa molecules studied at 600 MHz, $^{13}C-^{13}C$ NOE transfer and $^{13}C-^{13}C$ scalar transfer appear to be about equally efficient; therefore, we explored $^{13}C-^{13}C$ NOE experiments for the 19−21 kDa peptide-binding domains of the chaperone proteins Hsc and DnaK. We demonstrate here 2D H(CC)H and 3D (H)CCH versions of the following generalized 4D $^1H-^{13}C-^{13}C-^1H$ NOESY experiment: $p^H_{90} - \{t^H_1, 1/2J_{CH}\} - p^{C,H}_{90} - \{t^C_2, 1/3J_{CH}\} - p^C_{90} - \tau_M - p^C_{90} - \{t^C_3, 1/3J_{CH}\} - p^{C,H}_{90} - \{1/2J_{CH}\} - t^H_4$ The 4D experiment samples the frequencies of two adjacent carbon nuclei through two half semiconstant time periods $\{t^C_{2,3}, 1/3J_{CH}\}$ around the $^{13}C-^{13}C$ NOE mixing time τ_m transfers the magnetization from and to

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(4) We used the equation $E_{max} \cong \sin^2(\pi J_{CC}\tau_{max})\cos^2(\pi J_{CC}\tau_{max})\exp(-2\pi\nu_C^{1/2}\tau_{max})$ with a value of 1 Hz/kDa for the line width of the ^{13}C resonance ($\nu_C^{1/2}$) (25 °C); J_{CC} is 34 Hz.
(5) Side chain ^{13}C line widths can be dramatically reduced by protein perdeuteration, offering one solution to the transfer efficiency problem in HCCH-COSY. See also: Kushlan, D. M.; LeMaster, D. M. *J. Biomol. NMR* **1993**, *3*, 701. Farmer, B. T., II; Venters, R. A. *J. Am. Chem. Soc.* **1995**, *117*, 4187.
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(7) See Figure 1A in the following: Zeng, L.; Fischer, M. W. F.; Zuiderweg, E. R. P. *J. Biomol. NMR* **1996**, *7*, 157.
(8) Perdeuteration of the amino acid side chains shifts the curves in Figure 1a approximately 0.5 log unit to the left (i.e., NOE difference approaches maximum for molecules with τ_c of 10 ns). Clearly, perdeuteration could be of help, but gains in efficiency are likely to be offset by forfeiting the $^1H \rightarrow {}^{13}C$ polarization transfer and, more importantly, loss in recycling efficiency.
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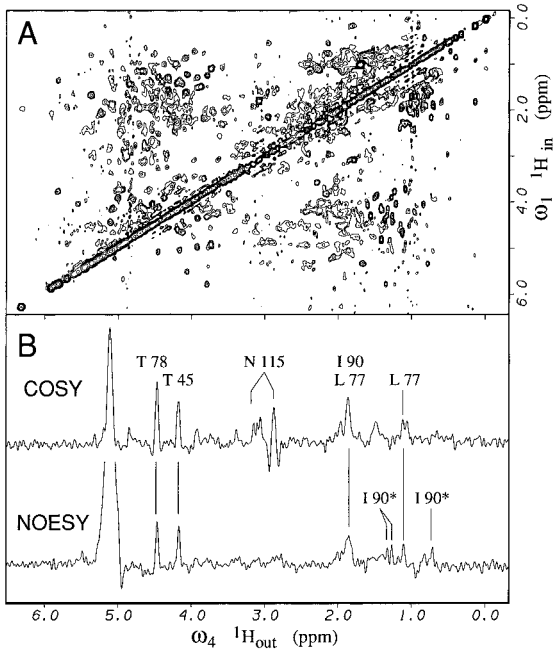

Figure 2. (A) Results of a two-dimensional H(CC)H-NOESY experiment, using a 1.4 mM sample of the 19 kDa peptide-binding domain of the molecular chaperone Hsc70 at 25 °C in ^2H_2O. A 600 MHz Bruker AMX instrument with a 5 mm triple-resonance gradient probe was used. The $^{13}C-^{13}C$ NOE mixing time was 1.0 s. Total experimental time was 36 h. (B) $^{13}C-^{13}C$ NOESY and $^{13}C-^{13}C$ COSY cross sections at 5.2 ppm. The H(CC)H COSY experiment[3] was recorded with the same number of scans and increments while using $^{13}C-^{13}C$ scalar transfer times of 9 ms. The assignments[14] are for Hα-Cα-Cβ-Hβ connectivities except for the spin diffusive I90 Hα-Cα-Cβ-Cγ-H_2 and Hα-Cα-Cβ-Cγ-H_3 which are marked with asterisks.

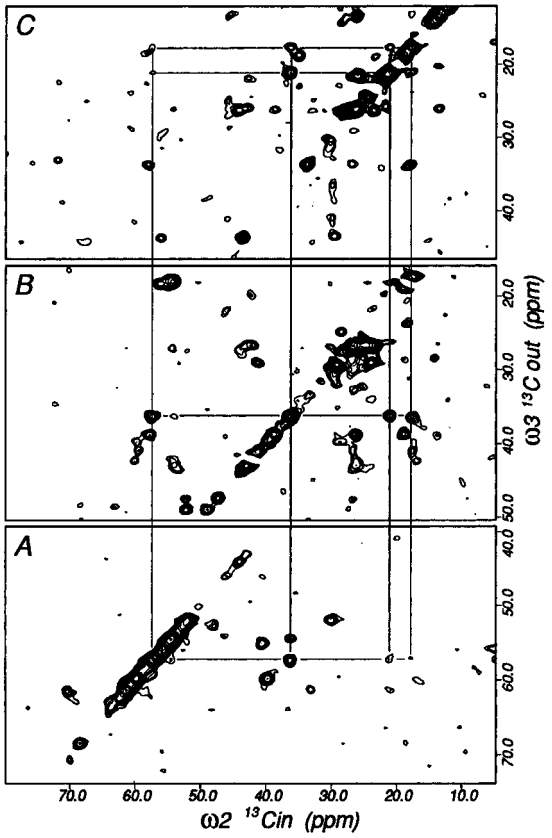

Figure 3. Results of a three-dimensional (H)CCH-NOESY experiment using a 1.2 mM sample of the 21 kDa peptide-binding domain of the molecular chaperone DnaK at 25 °C in ^2H_2O. A 600 MHz Bruker AMX instrument with a 5 mm triple-resonance gradient probe was used. $^{13}C-^{13}C$ planes (ω_2,ω_3) are shown at different ^1H frequencies (ω_4). A $^{13}C-^{13}C$ NOE mixing time of 1.0 s was used. The data set was 44 × 80 × 1024 hypercomplex points and was recorded with 16 scans per increment using a 1.0 s relaxation delay. Total experimental time was 150 h. In A, B, and C, the $^{13}C\alpha$, $^{13}C\beta$, $^{13}C\gamma_1$, and $^{13}C\gamma_2$ resonances of Val24 are traced[15] at the proton frequencies 5.29, 1.59, and 0.73, respectively (the $^1H\gamma_1$ and $^1H\gamma_2$ resonances are nearly degenerate). See Supporting Information and text for details.

the attached hydrogen nuclei using a half semiconstant time period $\{t_1^H, 1/2J_{CH}\}$ and a half-(R)INEPT sequence $\{1/2J_{CH}\}$.[10] We emphasize that ^{13}C magnetization is aligned along the $\pm z$ axis during the 1 s $^{13}C-^{13}C$ NOE mixing period τ_m while any remaining transverse magnetization (very little after 1 s) is dephased by pulsed-field gradients during that period. Figure 2A shows a 2D H(CC)H-NOESY experiment recorded with this pulse sequence; it is seen that the spectrum contains many $^{13}C-^{13}C$ NOE-mediated cross peaks. A cross section through the spectrum is shown in Figure 2B, together with an identical cross section through a 2D H(CC)H-COSY experiment. One indeed finds that $^{13}C-^{13}C$ dipolar and $^{13}C-^{13}C$ scalar transfer are about equally efficient for these conditions. We note that the spectra are complementary: narrow resonances are stronger in COSY, broader ones are stronger in NOESY. Figure 3 shows three $^{13}C-^{13}C$ planes from a 3D (H)CCH-NOESY experiment and gives the connectivity tracing for the residue Val24 of the 21 kDa protein DnaK. Panel B contains the one-bond $^{13}C-^{13}C$ NOEs $^{13}C\beta$ (37 ppm) → $^{13}C\alpha$ (57 ppm), $^{13}C\beta$ → $^{13}C\gamma_1$ (21 ppm), and $^{13}C\beta$ → $^{13}C\gamma_2$ (19 ppm) for this residue. The reciprocal one-bond $^{13}C-^{13}C$ NOE connectivities are indicated in panels A and C, where the presence of a small amount of spin diffusion is apparent (e.g., $^{13}C\gamma_1$ → $^{13}C\beta$ → $^{13}C\alpha$ and $^{13}C\gamma_1$ → $^{13}C\beta$ → $^{13}C\gamma_2$ in panel C). Peak picks in the ($^{13}C\beta$, $^{13}C\alpha$, $^1H\alpha$) region through the entire spectrum show that virtually all $^{13}C\alpha-^{13}C\beta$ crosspeaks for the DnaK domain are present. It is therefore demonstrated that it is possible to obtain good 3D (H)-CCH-NOESY spectra using a 5 mm sample of a 1.2 mM solution of a 21 kDa protein using a 600 MHz spectrometer.

According to our calculations, shown in Figure 1B, one expects that the sensitivity of the HCCH-NOESY experiment will only increase when larger proteins will be studied at larger magnetic fields. The NOE method should thus be considered for tracing side chain connectivities and may possibly replace the current HCCH-COSY and HCCH-TOCSY experiments for the study of larger molecules.

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Supporting Information Available: Derivation of an expression for the steady state NOE in a three-spin system; detailed description of the pulse sequence, gradient and phase cycles for the 4D HCCH NOESY experiment; experimental parameters for the 3D (H)CCH NOESY shown in Figure 3 (3 pages). See any current masthead page for ordering and Internet access instructions.

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