Relaxation-Optimized NMR Spectroscopy of Methylene Groups in Proteins and Nucleic Acids

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Abstract: A large fraction of hydrogens in proteins and nucleic acids is of the methylene type. Their detailed study, however, in terms of structure and dynamics by NMR spectroscopy is hampered by their fast relaxation properties, which give rise to low sensitivity and resolution. It is demonstrated that six different relaxation interference processes, involving $^1H\cdots^{15}C$ and $^1H\cdots^3H$ dipolar interactions and $^1H$ and $^{13}C$ chemical shift anisotropy, can be used simultaneously to mitigate these problems effectively. The approach is applicable to the majority of NMR experiments commonly used to study side chain and backbone conformation. For proteins, its efficiency is evaluated quantitatively for two samples: the third IgG-binding domain from Streptococcal Protein G and the protein calmodulin complexed with a 26-residue target peptide. Gains in both resolution and sensitivity by up to factors of 3.2 and 2.0, respectively, are observed for Gly residues at high magnetic field strengths, but even at much lower fields gains remain substantial. The resolution enhancement obtained for methylene groups makes possible a detailed analysis of spectral regions commonly considered inaccessible due to spectral crowding. For DNA, the high resolution now obtainable for $C^\gamma$ sites permits an $H^\gamma/H^\delta/H^\epsilon$-based sequential NOE assignment procedure, complementary to the conventional base-$H^\gamma/H^\delta/H^\epsilon$ pathway.

Introduction

In recent years, it has been shown that transverse-relaxation-optimized NMR spectroscopy (TROSY) methods can extend the application of NMR to considerably larger systems. To date, TROSY experiments have been described for optimizing observation of $^{15}N\cdots^1H$ amide groups, 1,2 aromatic $^{13}C\cdots^1H$ sites,3-5 and most recently $^{13}C\cdots^3H$ methyl groups.6 No such methods have been available for methylene groups, even though they represent 46% of the observable protons in proteins, and the $C^\gamma$ methylene group in nucleic acids represents a critical backbone element. Frequently, these methylene regions are difficult to access due to severe spectral crowding.

Here we present an approach, based on relaxation-optimized spectroscopy and multiplet simplification, that considerably improves both the resolution and sensitivity of methylene group NMR studies. The approach is applicable both to small molecules, where it is primarily the suppression of geminal $^1H\cdots^1H$ splittings that enhances spectral appearance, and biological macromolecules, where the benefits of the improved relaxation properties selected in our experiments further enhance spectral quality. Conceptually, the new approach is an extension of earlier E.COSY based technology,7 aimed at measurement of $^1H-^{15}C$ and $^1H-^3H$ dipolar couplings in proteins and nucleic acid methylene groups. The element introduced here for optimized NMR observation of methylene groups can be incorporated in many of today’s most common 2D and 3D NMR experiments. As an example, we show its incorporation in 3D $^{13}C$-separated NOESY, enabling observation of the sequential $H^\gamma\cdots^3H$ NOEs in DNA and thereby providing sequential assignment information. The experiments also provide access to the measurement of the geminal $^1H-^1H$ scalar and dipolar coupling, as well as both corresponding one-bond $^1H-^{13}C$ interactions.

Methods

All NMR experiments were carried out on Bruker DRX spectrometers, operating at $^1H$ frequencies of 500 or 800 MHz and equipped respectively with a cryogenic (500 MHz) or a regular (800 MHz) triple resonance pulsed field gradient probehead, optimized for $^1H$ detection.


All data sets have been processed with nmPipe. For resolution and sensitivity comparisons, no apodization of the time domain data was employed. Data were zero-filled by a factor of 2 in all dimensions prior to Fourier transformation.

Three different samples were employed. The first sample comprised 1.8 mM uniformly (89%) 13C,15N-labeled third IgG-binding domain from Streptococcal Protein G, hereafter referred to as GB3, dissolved in D2O, pH 5.6, 50 mM sodium phosphate (data were acquired at 10 °C). The second protein sample contained 1 mM uniformly (89%) 13C,15N-enriched calmodulin, complexed with an unlabeled 26-residue peptide comprising the calmodulin-binding domain of skeletal muscle myosin light chain kinase, hereafter referred to as CaMM13,11 dissolved in D2O, pH 6.8, 100 mM KCl (data acquired at 35 °C). The third sample corresponds to a 19-base pair DNA binding site of the transcription factors Oct-1 and Sox-2 in the HoxB1 promoter.12 One strand (purchased from Silantes GmbH, Munich, Germany) is uniformly 13C,15N-enriched (5 13C, 15N-labeled third IgG-binding domain; 5•CATGACAAAGACA•3) and forms a duplex with the unlabeled complementary DNA strand (5•CATTAG-CATGACAAAGACA•3). The DNA duplex was dissolved in D2O, at a concentration of 0.8 mM, in 10 mM sodium phosphate, 10 mM dithiothreitol, and 0.02% NaN3, pH 6.5 (data acquired at 35 °C).

Quantum chemical calculations of methylene proton chemical shielding tensors were carried out using the GIAO method as implemented in Gaussian03,13 the hybrid B3LYP functional, and the 6-311++G(3df,3pd) basis set on all atoms. Results reported are for the methylene group of the model system H:C–N(H)–C(O)–CH2–N(H)–CH3, which was constructed using standard bond lengths and angles, a N–C–C–N dihedral angle (psi) of 135°, and a C–C–N–C dihedral angle of −139°, to mimic a glycine residue in an antiparallel beta strand geometry. Calculations of the absolute chemical shielding tensors for Gly-³H² methylene protons yields the following parameters: for the H² proton (pro-R), ωR = 23.9 ppm, ωp = 28.3 ppm, ωq = 30.4 ppm; angles between the C–H vector and these three principal components are equal to 82.6°, 74.0°, and 89.8°, and angles between the H–H vector and these three principal components are equal to 47.3°, 42.8°, and 89.7°. For the H¹ proton (pro-S), the principal components are ωR = 23.9 ppm, ωp = 27.9 ppm, ωq = 33.6 ppm; angles between the C–H vector and these three principal components are equal to 82.7°, 73.7°, and 18.0°; and angles between the H–H vector and these three principal components are equal to 49.8°, 67.0°, and 49.0°. Calculations at the same level of theory on tetramethylsilane (B3LYP/6-311+G* optimized structure) yield a proton isotropic chemical shielding of 31.6 ppm.

Theoretical Section

We consider a system of three coupled spin-³/² nuclei (13C, ¹H, and ³H²), corresponding to an isolated methylene group. Each spin is assumed to be characterized by an axially symmetric chemical shielding tensor. The single quantum NMR spectrum of each proton consists of a doublet of doublets, corresponding to the spin states of the other ¹H and the ¹³C. Each of the four components of proton H¹ (Figure 1a) can be described by a single-transition operator (H₀), which can be represented as a combination of product operator terms, describing in-phase and antiphase transverse coherences H₁⁺, 2H₁⁺C₁, 2H₁⁺H₁⁺, 4H₁⁺C₁H₁⁺.

\[ H₀ = H₁⁺ + p \times 2H₁⁺C₁ + q \times 2H₁⁺H₁⁺ + pq \times 4H₁⁺C₁H₁⁺ \] (1)

where P and Q characterize the eigenstates of C₁ and H₁⁺ respectively: \( P, Q = \{ (+, +); (+, -); (-, +); (-, -) \} \), with “+” and “−” corresponding to \( m_z = +1/2 \) (½ spin state) and \( m_z = −1/2 \) (½ spin state), respectively. Correspondingly, \( p = 1 \) for \( P = +; p = −1 \) for \( P = −; q = 1 \) for \( Q = +; q = −1 \) for \( Q = − \). With the relative magnitude and the sign of couplings involved in a methylene spin system taken into account, the position of each multiplet component, corresponding to an

Figure 1. NMR transitions in a 1³CH₂ group. (a) Energy level diagram of an isolated CH²H group. Each eigenstate is defined by three signs “+” or “−” (with “+” for \( m_z = +1/2 \) and “−” for \( m_z = −1/2 \)), where the first sign corresponds to the ¹³C spin state and the latter two signs to H¹ and H². Long-dashed lines depict H¹ transitions, and short-dashed lines are ¹³C transitions, each labeled with its single transition operator, defined in eqs 1 and 3. (b) Schematic representation of the 2D multiplet pattern observed for an individual methylene ³H¹ in a fully coupled HSQC experiment. The diagram corresponds to JCH > 0, JCC < 0, and JCH = JCC = JHH. For JCH = JCC, C²⁺ and C²⁻ transitions have vanishing intensity (open ellipsoids). The chemical shift frequencies are marked δC and δH. Horizontal short-dashed lines and vertical long-dashed lines mark ¹³C and ¹H frequency transitions, respectively. The transverse relaxation rate for each transition is defined in eqs 2, 4, and 5. Italic characters “o” correspond to the panels of Figure 4 which display this multiplet component experimentally.

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individual $^1$H transition, is indicated in Figure 1b. As a result of interference between the different dipole–dipole and chemical shift anisotropy (CSA) relaxation mechanisms operating on a given $^1$H, different line widths are expected for each multiplet component. The relaxation rate of an individual transition is described by: \[ R(H^PQ) = \Gamma^{DD,DD}_{HHCH} + \Gamma^{DD,DD}_{HHJHJ} + \Gamma_{CSA,CSA} - p \times \Gamma^{CSADD}_{HHCH} - q \times \Gamma^{CSADD}_{HHCH} + pq \times \Gamma^{DD,DD}_{HHCH} \] where the first three terms correspond to dipolar ($\Gamma^{DD,DD}_{HHCH}$, $\Gamma^{DD,DD}_{HHJHJ}$, and $\Gamma_{CSA,CSA}$) correlation and CSA relaxation rates, and the last three terms correspond to cross-correlated relaxation between $^1$H CSA and $^1$H–$^1$H dipole–dipole interaction ($\Gamma^{CSADD}_{HHCH}$), $^1$H CSA and $^1$H–$^2$H dipole–dipole interaction ($\Gamma^{CSADD}_{HHCH}$), and dipole–dipole cross-correlated relaxation between $^1$H–$^1$C and $^1$H–$^2$H ($\Gamma^{DD,DD}_{CHCH}$). The four single-transition operators and corresponding relaxation rates for proton $^2$H are obtained by interchanging indices 1 and 2 in eqs 1 and 2.

The four $^1$H transitions (Figure 1a) are described by

\[ C^{PQ} = C_p + p \times 2CCH_{1}H_{z} + q \times 2CCH_{1}H_{z}^2 + pq \times 4CCH_{1}H_{z}^2 \] where $P$ and $Q$ now represent the eigenstates of $H_z$ and $H_z^2$, respectively. In a $^1$H-coupled HSQC spectrum of a methylene group, the two center components of the doublet-of-doublets, corresponding to transitions $C^+\cdot$ and $C^-\cdot$, are of opposite phase and cancel one another. The spectrum then appears as a doublet in the $^1$C dimension, consisting of the two observable transitions $C^+\cdot$ and $C^-\cdot$, separated by $\delta J_{CH}$ + $\delta J_{CCH}$. Analogous to eq 2, the relaxation rates of the $^1$C multiplet components are described by

\[ R(C^{PQ}) = \Gamma^{DD,DD}_{CHCH} + \Gamma^{DD,DD}_{CHCH} + \Gamma_{CSA,CSA} - p \times \Gamma^{CSADD}_{CHCH} - q \times \Gamma^{CSADD}_{CHCH} + pq \times \Gamma^{DD,DD}_{CHCH} \] Assuming isotropic rotational diffusion in the slow tumbling limit for a macromolecule with fast internal dynamics of moderate amplitude, the individual auto- and cross-correlated relaxation rates (eqs 2 and 4) are given by

\[ \Gamma^{DD,DD}_{HHCH} = \Gamma^{DD,DD}_{CHCH} = (\delta_{HH})^2 \tau_C S^2/5 \] \[ \Gamma^{DD,DD}_{HHJHJ} = (\delta_{HH})^2 \tau_C S^2/4 \] \[ \Gamma_{CSA,CSA} = 4(\delta_{HH})^2 \tau_C S^2/45 \] \[ \Gamma^{CSADD}_{CHCH} = 4(\delta_{HH})^2 \tau_C S^2/45 \] \[ \Gamma^{DD,DD}_{CHCH} = 2(\delta_{HH})^2 \tau_C S^2/15 \] where $A$, $M$, and $X$ denote the $C$, $H_1$, or $H_2$ spins, in arbitrary order, $\delta_{AM} = (\mu_{12} \gamma_{12} \gamma_{2D} / 8 \pi^2)^{1/2}$, $\delta_{AX} = (\gamma_B D_\alpha A_\alpha )^{1/2}$, $\theta_{CH}$ is the angle between the principal axis of interactions $\mu$ and $\mu'$, $\tau_C$ is the correlation time, $r_{AM}$ is the distance between nuclei $A$ and $M$, and $S^2$ is the squared generalized order parameter.16 Although, strictly speaking, a specific order parameter should be introduced for each pair of correlated interactions, in this study we assume a uniform value of $S^2 = 0.8$, frequently a reasonable approximation.17 The symbol $\Delta \sigma_\alpha$ denotes the chemical shielding anisotropy of spin $A$, defined as $\Delta \sigma_\alpha = (\sigma_{11} - (\sigma_{22} + \sigma_{33})/2)$, where $\sigma_{11}$ is the chemical shielding tensor component furthest from the isotropic shielding value.

**Results and Discussion**

As described in the theoretical section, the fully coupled (in both $^1$H and $^1$C dimensions) HSQC spectrum will show eight multiplet components for each methylene proton of an isolated $^{13}$CH$_2$ group. The different coefficients applicable for each multiplet component when calculating the total relaxation rate from the sum of the autorelaxation and cross correlation terms of eqs 2–5, $\Gamma_{CSA,DD}$, $\Gamma_{DD,DD}$, $\Gamma_{CSA,CSA}$, $\Gamma_{CSADD}$, $\Gamma_{DD,DD}$, and $\Gamma_{DD,DD}$, result in different line widths for each of these eight components, schematically indicated in Figure 1b. Analogous to the well-known $^1$H–$^{15}$N TROSY experiment,1 selection of the slowest relaxing component will enhance spectral resolution. As described below, such a selection can be made with high efficiency by a suitably optimized pulse scheme, yielding considerable resolution enhancement and even increases in sensitivity over conventional HSQC experiments. Below, the experiment is quantitatively analyzed for the three-spin system of Gly residues in uniformly $^{13}$C-enriched proteins, dissolved in D$_2$O. Application to other methylene spin systems in biomacromolecules, outside of the isolated-three-spin approximation, will also be presented.

**Methylene Spin-State-Selective Correlation.** Figure 2a shows the implementation of a 2D experiment that specifically selects one of the eight methylene HSQC multiplet components. This 2D CH$_2$-TROSY pulse scheme is very similar to the standard inverse heteronuclear two-dimensional sensitivity-enhanced HSQC experiment18 but lacks $^1$H decoupling during $t_1$ and $^1$C decoupling during $t_2$. It also uses modified timing durations in the final “Rance–Kay” element of the pulse sequence to accomplish CH$_2$ spin-state-selective coherence transfer or CH$_2$-S$^2$CT.

The pulse scheme starts with an initial INEPT transfer of $^1$H magnetization to $^1$C (time point $a$). An S$^2$E element,19,20 between time points $a$ and $b$, then selects the slowest relaxing, most downfield component of the $^1$C multiplet, $C^-\cdot$. The total S$^2$E duration, normally 1/(4$^2$J$_{CH}$), is $\approx 880 \mu s$, to account for the $^2$J$_{CH}$ separation between the outer multiplet components in the $^1$C dimension. The $^1$H$^+-\cdot$ magnetization component is eliminated by the 90$^9$ $^1$C pulse applied at time point $b$, which rotates it to the $z$-axis, whereas the $C^-\cdot$ magnetization remains in the transverse plane and evolves during $t_2$ with frequency $\delta_C$ + $^1$J$_{CH}$. This magnetization is then encoded by gradients $G_4$ and $G_5$. As pointed out above, $C^-\cdot$ can be written as the sum of the four product operator terms: $C^-\cdot = C_s - 2C_{CH} H_1^2 - 2C_{CH} H_2^2 + 4C_{CH} H_2 H_2^2$. The subsequent methylene spin-state-selective coherence transfer (point $d$ to $e$ in Figure 2a) transforms $C^-\cdot$ into the following four $^1$H single quantum coherences: $H_s$.

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Resolution Enhancement of Methylene Groups

Figure 2. Pulse schemes of CH$_2$-TROSY experiments. Narrow and wide bars indicate nonselective 90° and 180° pulses, respectively. Unless specified, pulse phases are $x$. Delay durations: $\Delta = 1/(3J_{CH})$; $t_1 = 0.34/(3J_{CH})$; $t_2 = 0.23/(3J_{CH})$. For selection of the downfield, $C^-$ component: $\phi_1 = -y$; $\phi_2 = (225^\circ, 45^\circ)$; $\phi_3 = x$; and $\phi_0 = x$ are used to select the $H^+$ component in the $^1$H dimension (see Table 1); $\phi_4 = (x, x, y, y)$, $\phi_5 = x$ and $\phi_{rec} = (x, -x, -x, x)$.

The experiment is recorded in the regular Rance–Kay manner; for each $t_1$ increment, two FID’s are acquired, one with $G_5$, $G_6$, and $\phi_3$ inverted, and stored separately. Field gradients are sine-bell shaped with durations $G_1$ to $G_10$ of 1, 2, 0.25, 1, 1, 0.2, 0.3, 0.35, 0.153, 0.6 ms; amplitudes of 10, 18, 12, 30, 30, 10, 12, 30, 18 Hz/cm; and directions $x$, $y$, $x$, $-z$, $z$, $z$, $-z$, $x$, $y$. (a) 2D CH$_2$-TROSY experiment, in the nonconstant-time mode (i.e., optimal for Gly). The shaped $^{13}$C$_{iso}$ pulse has a REBUMP profile (3.1 ms duration at 800 MHz for a 6 ppm bandwidth inversion). $^{13}$C 180° pulses (carrier at 177 ppm) are sine-bell-shaped and have durations of 180 $\mu$s. (b) Pulse scheme of the 2D CH$_2$-TROSY and 3D CH$_2$-TROSY-NOESY adapted for nucleic acids. The shaped $^{13}$C$_{iso}$ pulse has been generated by coadding two REBUMP profiles, designed to refocus selectively two separate frequency regions: 65–70 ppm and 38–43 ppm (3.75 ms pulse duration at 800 MHz). Homonuclear band-selective $^{13}$C$_{iso}$ effective decoupling over the 76–89 ppm frequency range is achieved by means of a train of 180° adiabatic pulses (WURST-4 created with the Bruker shape tool, $Q_0$ factor = 4, for an inversion bandwidth of $\pm 1300$ Hz. maximum radio frequency field strength $\gamma B_2/2\pi = 804$ Hz, 5.25 ms duration at 800 MHz); in a p5m4 composite decoupling sequence ($2 \times (0, 150, 60, 150, 0), 2 \times (180, 330, 240, 330, 180)$). $^{13}$C decoupling during acquisition is only applied for the 3D experiments, using a GARP decoupling sequence with $\gamma B_2/2\pi = 4.2$ kHz. Quadrature detection in $^{13}$C ($t_1$) and $^1$H ($t_2$) dimension is achieved by recording four separate free induction decays or FIDs ($E_1$ to $E_4$) per pair of ($t_1, t_2$) values, with the following phases and gradient settings: $E_1 = (\phi_1 = x, \phi_2 = y, G_4, G_3); E_2 = (\phi_1 = -x, \phi_2 = x, -G_4, -G_3); E_3 = (\phi_1 = x, \phi_2 = y, G_4, G_3); and E_4 = (\phi_1 = -x, \phi_2 = y, -G_4, -G_3)$. Prior to Fourier transformation, the FIDs are rearranged as follows: $E_1' = (E_1 + E_2); E_2' = (E_1 - E_2); E_3' = (E_3 + E_4); and E_4' = (E_3 - E_4)$. Corresponding Bruker pulse programs and NMRpipe macros are available at http://spin.niddk.nih.gov/bax/.

2$^1H^1C_3$, $-2^1H^1C_2$, $-4^1H^1C_2$, $-3^1H^1C_2$. With identical values assumed for the two bond couplings, $J_{CH^1} = J_{CH^3} = J_{CH^2}$, the transfer efficiency in this CH$_2$-S'CT element is the same for generation of $H^1_{CH}^1(t_1, t_2)$ and $-4H^1_{CH}2(t_1, t_2)$ terms and described by a factor $f(t_1, t_2)$:

\[
f(t_1, t_2) = \cos(\pi J_{CH^1} t_1) \sin(\pi J_{CH^1} t_2) + \sin(\pi J_{CH^1} t_1)
\]

Generation of $2H^1_{CH}C(t_1, t_2)$ and $-2H^1_{CH}C(t_1, t_2)$ also carries a common prefactor:

\[
g(t_1, t_2) = \sin(\pi J_{CH^1} t_1) \cos(\pi J_{CH^1} t_2) \cos(\pi J_{CH^2} t_2) + \cos(\pi J_{CH^1} t_2) \sin(\pi J_{CH^1} t_2)(1 + \sin^2(\pi J_{CH^1} t_2))
\]

If $t_1$ and $t_2$ delays are chosen such that $f(t_1, t_2) = g(t_1, t_2)$, cancellation occurs for signals corresponding to transitions $H^+, H^-$, and $H^+$ (Figure 3a). In contrast, the terms contributing to $H^+$ magnetization add constructively, yielding a $^1$H multiplet in which only the most upfield component has nonzero intensity (Figure 3a). Although a continuous range of ($t_1$, $t_2$) values can satisfy $f(t_1, t_2) = g(t_1, t_2)$ (Figure 3b), the solution ($t_1 = 0.34/1J_{CH^1}; \ t_2 = 0.23/1J_{CH^1}$) corresponds to the highest transfer efficiency (Supporting Information Figure S1), and use of these values maximizes the sensitivity of correlating $C^-$ and $H^+$ transitions.

Figure 4 demonstrates that each of the eight $^1$H–$^{13}$C multiplet components for a given methylene proton can be selected separately with the pulse scheme of Figure 2a. The correlations correspond to the C$^3$H$^3$ spin pair of Gly-41 in GB3. Different correlations in the $^1$H dimension are selected by changing phases $\phi_0$ and $\phi_3$ in the CH$_2$-S'CT element in the manner described in Table 1. These phases control the signs of the ($t_1$, $t_2$)-dependent transverse $^1$H magnetization terms, generated by the CH$_2$-S'CT element: $H^1_{CH}^1(t_1, t_2), 2H^1_{CH}^1(t_1, t_2), 2H^1_{CH}^2(t_1, t_2), 2H^1_{CH}^2(t_1, t_2)$. So, these terms can be individually inverted, without affecting their absolute magnitude. Next to selection of the desired proton transition by $\phi_0$ and $\phi_3$, the phase $\phi_3$ of the $^{13}$C 90° pulse preceding the S'E element controls selection of the desired $^{13}$C multiplet component, evolving during $t_1$. Analogous to previously proposed TROSY experiments for $^{13}$N–$^1$H and aromatic $^{13}$C–$^1$H spin systems, the

steady-state Boltzmann 13C polarization can be added to the magnetization transferred from 1H by appropriate selection of the phase φ1 in Figure 2, thereby enhancing sensitivity. For each of the eight multiplet components, the optimized combinations of radio frequency phases are given in Table 1.

Incomplete suppression of multiplet components not selected by a given combination of phases in Table 1 can arise from deviations from the τ1 = 0.34/JCH and τ2 = 0.23/JCH condition, which cannot be met simultaneously for the experimental range of JCH values (referred to as 1JCH mismatching).

Other causes of incomplete suppression include differential relaxation, the finite lifetime of the passive spin state, and, in particular for the methylene case, evolution resulting from the relatively large 2JHH coupling. The selection efficiency of the S1E element in the presence of 1JCH mismatching has already been well documented. Simulation31 of effects induced by the neglected 2JHH evolution or due to 1JCH mismatch during the CH2-S3CT element demonstrate that the intensity of multiplet components not selected by any given phase cycle combination of Table 1 will be ≤0.6% (for 2JHH = −18 Hz) and ≤0.1% (for ΔJCH/JCH ≤ 0.1). Because the transfer delays τ1 and τ2 are relatively short, the effect of passive spin state flips or differential relaxation during the final CH2-S3CT element is relatively small and does not result in significant breakthrough of unselected components. Perhaps surprisingly, initial experiments using 500-µL sample volumes in regular NMR tubes revealed that B1-field inhomogeneity can result in detectable spurious peaks (up to 4% of the selected component). However, in all tested cases (using 500 and 600 MHz cryogenic probeheads and 600 and 800 MHz room-temperature probes), this artifact is reduced below 1% by restricting the sample height, using a 280-µL volume in a thin-wall Shigemi microcell. One-dimensional cross sections, shown in Figure 4, confirm that no selection artifacts are observable at the noise threshold. However, accurate pulse calibrations remain critical to achieve clean selection: simulated and experimental results indicate that 1H or 13C pulse miscalibration by as little as 5% can result in selection artifacts of up to 7%.

Whereas, in standard 1H-coupled HSQC experiments, correlations involving the C− and C+− transitions are not observed, they can potentially lead to undesirable correlations in the CH2-TROSY experiment. It can be shown that such artifacts arise from the 13C Boltzmann polarization (vide infra, eq 8). In this case, the CH2-S3CT element converts the CH2-TROSY experiment. It can be shown that such artifacts arise from the 13C Boltzmann polarization. TheCH2-S3CT element demonstrates that the intensity of multiplet components not selected by any given phase cycle combination of Table 1 will be ≤0.6% (for 2JHH = −18 Hz) and ≤0.1% (for ΔJCH/JCH ≤ 0.1). Because the transfer delays τ1 and τ2 are relatively short, the effect of passive spin state flips or differential relaxation during the final CH2-S3CT element is relatively small and does not result in significant breakthrough of unselected components. Perhaps surprisingly, initial experiments using 500-µL sample volumes in regular NMR tubes revealed that B1-field inhomogeneity can result in detectable spurious peaks (up to 4% of the selected component). However, in all tested cases (using 500 and 600 MHz cryogenic probeheads and 600 and 800 MHz room-temperature probes), this artifact is reduced below 1% by restricting the sample height, using a 280-µL volume in a thin-wall Shigemi microcell. One-dimensional cross sections, shown in Figure 4, confirm that no selection artifacts are observable at the noise threshold. However, accurate pulse calibrations remain critical to achieve clean selection: simulated and experimental results indicate that 1H or 13C pulse miscalibration by as little as 5% can result in selection artifacts of up to 7%.

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represents only ca. 2% of the selected correlation in the regular CH2-TROSY spectrum. Use of longer delays, which still satisfy the equality $f(t_1, t_2) = g(t_1, t_2)$ (Figure 3b), does not cause the intensity of this artifact even further (Supporting Information, Figure S2). Total cancellation can be obtained for $t_1 = 0.50/\text{J}_{\text{CH}}$ and $t_2 = 0.25/\text{J}_{\text{CH}}$ but would occur at the expense of a 20% drop in sensitivity (Supporting Information, Figure S1) and an increase of other sources of artifacts. Therefore, as a compromise between sensitivity and artifact intensity, values of $t_1 = 0.4/\text{J}_{\text{CH}}$ and $t_2 = 0.24/\text{J}_{\text{CH}}$ are used. With these delays, no artifact larger than 1% is observed and the experiment still retains $\geq 95\%$ of its maximum sensitivity.

**High Resolution $^1\text{H}$$-^{13}\text{C}$ Spectra.** As expected, intensities and line widths of the eight multiplet components of methylene $^1\text{H}$$-^{13}\text{C}$ correlation spectra vary considerably (Figure 4). The correlation between transitions $C^−$ and $H^+$ exhibits the most favorable relaxation properties (Figure 4b). The longer relaxation time of $H^+$ compared to $H^+$ indicates that at the position of proton $H^+$, the sum of the dipolar fields corresponding to $^{13}\text{C} = |\alpha\rangle$ and $^{13}\text{C} = |\beta\rangle$ decreases the “local field” of the $^1\text{H}$ CSA term. Similarly, the longer relaxation time of $C^−$ compared to $C^−$ indicates that, at the $C$ position, the sum of the dipolar fields decreases the “local field” of the $^{13}\text{C}$ CSA term when both protons are in the $|\beta\rangle$ spin state. Therefore, optimal resolution is obtained when the CH2-TROSY experiment selects this correlation.

As an example, Figure 5 compares the Gly region of such a CH2-TROSY spectrum, recorded for the 20 kDa CaM/M13 complex, with a regular sensitivity-enhanced HSQC experiment, optimized for methylene correlations.18,32 Comparison of $^1\text{H}$ line widths in these two spectra highlights the increased resolution.

We define a resolution enhancement factor $R_{\text{R}}$ as the ratio of the NMR signal line width measured in the HSQC and in the CH2-TROSY spectra. The resolution enhancement factor in the

$$\lambda_{\text{H}} = \frac{R_{\text{H}} + \pi \times |^2J_{\text{HH}}|}{R_{\text{H}} + R_{\text{H}}^{\text{DD,DD}} + R_{\text{H}}^{\text{CA,DD}} + R_{\text{H}}^{\text{DD,CA}}}$$ (7a)

where $R_{\text{H}}$ is the proton auto-relaxation rate: $R_{\text{H}} = R_{\text{H}}^{\text{DD,DD}} + R_{\text{H}}^{\text{DD,DD}} + R_{\text{H}}^{\text{CA,DD}} + R_{\text{H}}^{\text{DD,CA}}$. Each of the three pertinent cross-correlated rates, $R_{\text{H}}^{\text{DD,DD}}$, $\Gamma_{\text{DD,DD}}$ and $\Gamma_{\text{DD,DD}}^{\text{DD,CA}}$ can be extracted from the difference of $^1\text{H}$ line widths for suitably chosen pairs of resonances (eq 2). Analysis of spectra recorded for the CaM/M13 complex, at 35 °C and at a $^1\text{H}$ frequency of 800 MHz (data not shown), yields average values of $R_{\text{H}}^{\text{DD,DD}} = 27.4 \pm 3.2$ Hz, $\Gamma_{\text{DD,DD}} = 6.2 \pm 3.9$ Hz, and $\Gamma_{\text{DD,DD}}^{\text{DD,CA}} = -0.6 \pm 3.9$ Hz. Therefore, for moderate size proteins, the two most important factors for the line narrowing effect in the $^1\text{H}$ dimension are the dipole–dipole cross-correlated rate $R_{\text{H}}^{\text{DD,DD}}$ and the suppression of the $^2J_{\text{HH}}$ splitting (17–18 Hz for Gly). Whereas the absolute line narrowing caused by removal of the $^2J_{\text{HH}}$ splitting remains constant, the contribution from the cross-correlated relaxation increases with the correlation time and thereby the size of the protein. For the CaM/M13 complex, the experimentally determined resolution enhancement factor is $\lambda_{\text{H}} = 2.30$, with $\lambda_{\text{H}} = 1.54$ and $\Gamma_{\text{H}} = 1.49$. For the tetrahedral geometry of the Gly methylene groups, substitution of our experimental value of $R_{\text{H}}^{\text{DD,DD}}$ into eq 5e yields $\tau S^2 = 6.74$ ns. With a generalized order parameter $S^2 = 0.8$ for Gly-Cα assumed, this corresponds to $\tau = 8.4$ ns, in good agreement with previous measurements.33 No prior experimental data for the Gly-$^1\text{H}$ chemical shielding tensor magnitude or orientation...
are available to confirm the measured cross-correlated relaxation rates, and instead we use the results from DFT calculations (see Methods). For $\tau_S$ $S^2 = 6.74$ ns and DFT-derived $^1$H shielding tensors, the predicted cross-correlated relaxation rates averaged over the two methylene protons ($\Gamma_{^1H,^1H^*} = +6.56$ Hz and $\Gamma_{^1H,^1H^*} = -0.17$ Hz) are in very good agreement with experimental results ($\Gamma_{^13C,^1H^*} = +6.2$ Hz and $\Gamma_{^13C,^1H^*} = -0.6$ Hz).

In the $^{13}$C dimension, the resolution enhancement factor $\lambda_C$ is defined as the ratio of the HSQC line width over that in CH$_2$-TROSY:

$$\lambda_C = \frac{R_C + \Gamma_{^1H,^1H^*}}{R_C + \Gamma_{^1H,^1H^*} + \Gamma_{^1H,^1H^*} + \Gamma_{^1H,^1H^*}} (7b)$$

where $R_C$ is the carbon autorelaxation rate: $R_C = \Gamma_{^1H,^1H^*} + \Gamma_{^1H,^1H^*} + \Gamma_{^1H,^1H^*}$. Again assuming tetrahedral geometry and $\tau_S$ $S^2 = 6.74$ ns, calculation of the dipole–dipole cross-correlation rate yields $\Gamma_{^1H,^1H^*} \approx -20.5$ Hz. Although this corresponds to a ca. 25% decrease in $^{13}$C transverse relaxation rate compared to $R_C$, $\Gamma_{^1H,^1H^*}$ affects the two $^{13}$C multiplet components, $C^-$ and $C^+$, observed in HSQC and CH$_2$-TROSY identically. In contrast, the two CSA-dipole cross-correlated rates $\Gamma_{^1H,^1H^*}$ and $\Gamma_{^1H,^1H^*}$ broaden the upfield $^{13}$C multiplet component and narrow the downfield, $C^-$ transition, observed in the CH$_2$-TROSY experiment. For calmodulin Gly residues at 800 MHz, measurement of the decay rates of $C^-$ and $C^+$ yields $\Gamma_{^1H,^1H^*} + \Gamma_{^1H,^1H^*} = -17 \pm 5$ Hz. This corresponds to a $^{13}$C resolution enhancement factor $\lambda_C = 1.4$.

Assuming $\theta_{^1H,^1H^*} = \theta_{^1H,^1H^*}$, the value of $\lambda_C \times P_2(\cos \beta_{^1H,^1H^*})$ is determined from eq 5d and the experimental $\Gamma_{^1H,^1H^*}$ + $\Gamma_{^1H,^1H^*}$ rate. This yields $\lambda_C \times P_2(\cos \theta_{^1H,^1H^*}) = -25$ ppm, in good agreement with the experimental CSA values measured by solid-state NMR for Gly-C$_n$.

Finally, the total resolution enhancement factor of the CH$_2$-TROSY over the HSQC experiment is $\lambda_T = \lambda_C \times \lambda_H = 3.2$. With the relative sign of each cross-correlated relaxation rate involved taken into account, the CH$_2$-TROSY experiment makes it possible to benefit from all six relaxation interference effects to reduce the decay rate of the selected multiplet component. Cross-correlated relaxation rates increase with the molecular correlation tumbling time $\tau_C$. For the CSA values reported above, the resolution enhancement resulting from interference effects, $\lambda_C \times \lambda_H$, becomes larger than $\lambda_T$ for molecules with $S^2_{TC} > 5.3$ ns. CSA-dipole cross-correlated relaxation increases linearly with magnetic field strength, whereas $J_{HH}$ coupling and, to a good approximation, dipole–dipole cross-correlated relaxation rates are field-independent. However, considering that the magnitudes of $^1$H and $^{13}$C CSA are moderate for aliphatic groups, suppression of the $J_{HH}$ splitting and dipole–dipole cross-correlation are the dominant factors behind the increased resolution. Therefore, the CH$_2$-TROSY experiment remains quite efficient at low field: a resolution enhancement factor of $\lambda_R = 2.8$ is observed for the CaM/M13 complex at 500 MHz, compared to the above-mentioned $\lambda_T = 3.2$ at 800 MHz (Table 2).


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**Figure 5.** Comparison of (a) HSQC and (b) CH$_2$-TROSY spectra of Gly C$_n$H$_2$ groups in $^{13}$C-enriched calmodulin, complexed with unlabeled M13 peptide. Correlations are labeled by residue number. Both experiments were optimized for methylene detection, with the $\tau_1$ delay of the Rance–Kay element in the HSQC experiment adjusted to 1/(8 $\delta_{^1H}$). Spectra were recorded at 800 MHz, 35 °C, with a relaxation delay of 2.5 s and total measuring times of 2.3 h each. Identical acquisition and processing parameters were used: time domain matrices of 192$^* \times 640$ data points, with acquisition times of 96 ms ($t_1$) and 80 ms ($t_2$), no apodization of the time domain data. Spectra are plotted at identical contour levels, and correlations are labeled by residue number. Inset cross sections, taken at the position of the dashed lines, correspond to Gly-23. The average resolution enhancement obtained with CH$_2$-TROSY is $\lambda_T = 3.2$, while sensitivity is increased by the factor $\lambda_S = 2$.

**Table 2.** Average Observed Gly C$_n$H$_2$ Resolution and Sensitivity Enhancement Factors in a Calmodulin/Peptide Complex$^*$

<table>
<thead>
<tr>
<th>$\delta_0$ (T)</th>
<th>$\lambda_R$</th>
<th>$\lambda_T$</th>
<th>$\lambda_S$</th>
<th>$\lambda_H$</th>
<th>$\lambda_C$</th>
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<td>11.75</td>
<td>2.3</td>
<td>1.2</td>
<td>2.8</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>18.8</td>
<td>2.3</td>
<td>1.4</td>
<td>3.2</td>
<td>2.0</td>
<td></td>
</tr>
</tbody>
</table>

$^*$ Data at 35 °C for a ($^{13}$C, $^{15}$N)-labeled sample of the 20 kDa CaM/M13 complex in D$_2$O. $^1$The recycle delay, optimized for maximum sensitivity, is 1.8 s at 11.75 T and 2.5 s at 18.8 T. $^2$The resolution enhancement factors $\lambda_R$ and $\lambda_S$ are defined as the ratios of the $^1$H ($\lambda_R$) and the $^{13}$C line widths in the regular HSQC and CH$_2$-TROSY spectra. The total resolution enhancement factor, $\lambda_T$, equals the product of $\lambda_H$ and $\lambda_C$. The sensitivity enhancement factor, $\lambda_S$, is the ratio of the intensity in the CH$_2$-TROSY and regular HSQC spectra, recorded with identical acquisition and processing parameters.

**Sensitivity Enhancement.** Compared to the regular HSQC experiment only one-half of the initial $^1$H polarization is retained after the $S^2$E filter ($\lambda_{S^2E} = 0.5$). This loss is partially compensated
by the addition of the $^{13}$C Boltzmann polarization, which contributes to the detected signal in the CH$_2$-TROSY experiment, as discussed previously. At the end of the INEPT element (time point $a$, Figure 2a) the signal intensity is described by the following:

$$I(\delta) = M_0(\delta) \times [2C_H + 2C_H^2] + M_C(\delta) \times [C]_0,$$

where $M_0(\delta)$ is the initial Boltzmann magnetization for nucleus $X$, using a recycling delay, $\delta$. $I(\delta)$ can be decomposed in terms of single-transition operators $C^0$ (eq 3):

$$I(\delta) = C^- \left( \frac{M_C(\delta)}{4} + \frac{M_H(\delta)}{2} \right) + C^+ \left( \frac{M_C(\delta)}{4} - \frac{M_H(\delta)}{2} \right) + M_C(\delta) \frac{C^+ + C^-}{4} \tag{8}$$

Only $C^-$ is selected in the CH$_2$-TROSY experiment. Because $M_X$ is directly proportional to the gyromagnetic ratio, $\gamma_X$, of $X$ ($\gamma_H/\gamma_C \approx 4$; i.e., $M_H/M_C \approx 4$), eq 8 indicates that a 12.5% signal increase is expected when the $^{13}$C Boltzmann magnetization is added. A larger sensitivity gain is expected under nonequilibrium, steady-state conditions for the case where $T_1(\text{H}) > T_1(\text{C})$.2,24

The dependence of signal contributions resulting from $^1$H and $^{13}$C steady-state polarization as a function of the recycle delay, $\delta$, has been measured for the calmodulin Gly residues at a 18.8-T (800 MHz $^1$H frequency) magnetic field strength (Supporting Information, Figure S3). Optimal signal-to-noise per unit of measuring time is obtained for $\delta \approx 2.5$ s. At this interscan delay, the $^{13}$C Boltzmann magnetization corresponds to a signal increase of 33% over the case where only the magnetization transferred from $^1$H is selected. In practice, shorter interscan delay times are frequently used to complete all required phase cycling steps in a finite amount of available measurement time. For a typical $\delta \approx 1$ s duration, the $^{13}$C Boltzmann component increases the signal by 50%; i.e., the total amount of observed $^1$H magnetization becomes 75% of that in a regular HSQC experiment.

The line narrowing effect resulting from cross-correlated relaxation in both $^1$H and $^{13}$C dimensions also contributes to increased intensity of the detected signal in the CH$_2$-TROSY experiment. However, if the acquisition time in the $^{13}$C dimension were adjusted to a fixed multiple of the effective $^{13}$C magnetization decay constant, the slower decay of $^{13}$C magnetization has a negligible effect on the sensitivity of the experiment per unit of measuring time. In contrast, in the directly detected $^1$H dimension, where longer sampling does not increase the total time of the experiment, sensitivity per unit of measuring time increases with the square root of the obtained resolution enhancement factor, again assuming that the acquisition time equals a fixed multiple of the decay constant in the two experiments. In practice, sensitivity comparisons of regular and TROSY type experiments are commonly carried out under conditions where identical sampling times are used for the regular and the TROSY-type experiments.2,35 A comparison which increases the apparent sensitivity in the TROSY-type experiment, but which obviates calculation of the precise, residue-dependent timing parameters needed for optimizing each experiment. For simplicity, we therefore adhere to this common practice and compare data sets that have identical sampling durations in both $^1$H and $^{13}$C dimensions and process the spectra without any apodization of the time domain data.

Under these conditions, the expected sensitivity enhancement factor coming from favorable relaxation properties of the CH$_2$-TROSY experiment equals $2 \times \lambda_C \times \lambda_H$, where $\lambda_C$ and $\lambda_H$ are defined in eq 7, and where the factor 2 is due to the suppression of the $^2$JHH splitting, assuming $^2$JHH is resolved in the HSQC spectrum. As discussed above, experimentally we find $\lambda_C = 1.4$ and $\lambda_H = 1.49$ for the Gly-$^{13}$C-$^2$H$_2$ spin system at $B_0 = 18.8$ T. However, an additional factor, $\lambda_T$, needs to be defined to account for the reduced transfer efficiency of the CH$_2$-$^3$CT transfer relative to a methylene-optimized, sensitivity-enhanced Rance–Kay element. This factor depends on the delays $t_1$ and $t_2$ (Supporting Information Figure S1) and has a maximum for $t_1 = 0.34/J_{CH}$ and $t_2 = 0.23/J_{CH}$ ($\lambda_T = 0.85$). With all of the described effects taken into account ($^{13}$C spin state selection, use of $^{13}$C Boltzmann polarization, cross-correlated relaxation, proton–proton decoupling enhancements, and transfer efficiency), the total sensitivity enhancement is then given by

$$\lambda_S = \lambda_{SE} \times \left( \frac{M_C(\delta) + M_H(\delta)}{M_H(\delta)} \right) \times 2 \times \lambda_C \times \lambda_R \times \lambda_T \tag{9}$$

yielding a value of $\lambda_S = 2.2$, in good agreement with a value of 2.0, observed experimentally at 800 MHz (Table 2). The 10% discrepancy between predicted and experimentally determined sensitivity enhancements is likely to result from two factors neglected in the above calculation: the effect of relaxation during the very short SE filtering delay and partial overlap of the two $^1$H doublet components in the reference HSQC spectrum. This partial overlap increases the apparent HSQC intensity and, therefore, reduces the observed gain of the CH$_2$-TROSY experiment. When it is considered that much of the gain in resolution and sensitivity results from dipole–dipole cross correlation effects, which are independent of magnetic field strength, the CH$_2$-TROSY experiment remains advantageous across the entire range of commonly used magnetic field strengths. Indeed, a sensitivity enhancement factor of $\lambda_S = 1.6$ was observed for the CaM/M13 sample at 500 MHz, only 20% less compared to the gain observed at 800 MHz.

**Effect of Nongeminal $^1$H–$^1$H Interactions.** Above, discussions focused on an isolated three-spin system, exemplified through the study of Gly methylene groups. However, the experiment is directly applicable to all methylene sites in macromolecules. For such nonsolated methylenes, remote $^1$H–$^1$H interactions need to be taken into account when evaluating the merits of the CH$_2$-TROSY experiment. Both passive scalar interaction, $^3$JHH, and dipolar $^1$H–$^1$H relaxation with the additional protons will broaden the $^1$H signal, whereas the effect of the additional protons on the $^{13}$C decay will be negligible compared to that of its directly bonded protons. For the methylene protons, the difference in $^1$H line width between the CH$_2$-TROSY and regular HSQC experiments is not affected by the additional presence of neighboring protons, but the relative resolution and sensitivity gain decrease as the remote protons broaden the $^1$H line width in both types of experiments by the same amount.

The effect of protons in the vicinity of a methylene group can be described by adding the following terms to both the

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The intensity ratio observed in CH2-TROSY versus HSQC spectra

\[ \Sigma_{J_{HH}} \text{for} \text{the} \text{case of isolated methylenes}. \text{Also, the additional} \text{H-} \text{H dipolar interactions increase the effective} \text{H longitudinal relaxation rate of methylene protons, without significantly affecting the relaxation rate of} \text{13C, thereby reducing the fractional contribution of the Boltzmann} \text{13C polarization.}

To evaluate the enhancement offered by the CH2-TROSY experiment for nonisolated methylene groups experimentally, we recorded CH2-TROSY and regular 1H-13C HSQC spectra for GB3 at 800 MHz and 10 °C. The GB3 protein (56 residues) is much smaller than calmodulin and yields resolved correlations for most of its methylene resonances, in both the CH2-TROSY and HSQC spectra (Supporting Information, Figure S4). The spectra can be recorded either in the constant-time (CT) mode (using a CT 13C evolution period of 1/1, TCT ≈ 28 ms) or in the regular manner. If the CH2-TROSY spectrum is recorded in the CT mode, 14CH2 couplings (n ≥ 2) are not depopulated and cause some attenuation at the end of the CT evolution period: for example, JCH = 7 Hz results in a 20% loss of magnetization. On the other hand, as is the case for 3D 15N TROSY-based experiments,37 the favorable 13C relaxation properties apply for the full duration of the CT period, T, whereas in the regular CT-HSQC favorable relaxation applies to the two multiplet components for durations of T − t1/2 and t1/2. This changes the intensity of the CH2-TROSY signal by a factor of 2(ΓCS,DD + ΓCS,DD) / T((1 − exp − 2(ΓCS,DD + ΓCS,DD)) / T) relative to that in the HSQC experiment. In the CT mode, this factor should substitute for JCH in eq 9 when calculating the sensitivity enhancement factor, δS. This δS factor increases with the size of the molecule, and its magnitude becomes larger than the enhancement obtained for regular, non-CT acquisition mode (eq 7b) when Sτc ≥ 5.7 ms, assuming a regular CT duration of T = 28 ms. For side chain methylene carbons, we find that the intensity ratio observed in CH2-TROSY versus HSQC spectra is also strongly influenced by the amplitude of internal dynamics of the methylene group, with more internal motion resulting in a smaller value of δS.38 In constant-time experiments, improved spectral appearance therefore mainly results from the line narrowing obtained in the 1H dimension. This latter effect depends strongly on the number of protons geminal to the methylene carbon in question.39 CH2 methylenes of Cys, Ser, Asp, Asn, Phe, Tyr, Trp, and His each have only a single proton (Hβ) that is J-coupled to Hβ. For these residues, the gain in 1H resolution is, on average, δH = 1.7 (Table 3). Smaller gains are obtained for CH2 methylenes of Met, Glu, Gln, Arg, and Lys residues (Table 3), where three vicinal protons have JHH and dipolar interactions with Hβ.

When comparing C2′ to Cα methylene groups in DNA, we also find smaller enhancements in resolution and sensitivity: δH = 1.6 vs 1.9 and δS = 1.0 vs 1.3 (Table 3). The disappearance of a sensitivity gain is again consistent with the topology of

<table>
<thead>
<tr>
<th>CH2 type</th>
<th>Cα</th>
<th>C, S, D, N, F, Y, W, Hβ</th>
<th>M, Q, E, K, Rβ</th>
<th>C2′</th>
<th>Cα′</th>
</tr>
</thead>
<tbody>
<tr>
<td>δH</td>
<td>2.3</td>
<td>1.7</td>
<td>1.4</td>
<td>1.6</td>
<td>1.9</td>
</tr>
<tr>
<td>δS</td>
<td>1.7</td>
<td>1.0</td>
<td>0.8</td>
<td>1.0</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Electrical data recorded at 800 MHz, using the pulse sequence of Figure 2a for samples of 15C, 15N-enriched GB3 (T = 10 °C), and using the pulse sequence of Figure 2b for a 19-basepair DNA oligomer (T = 35 °C), both in D2O. The resolution enhancement factor, δH, corresponds to the average 1H linewidth in the regular HSQC experiment divided by that in the CH2-TROSY experiment. The sensitivity enhancement factor, δS, is the ratio of the intensity in the CH2-TROSY spectrum divided by that in the regular HSQC spectrum, recorded with identical acquisition and processing parameters.3 One-letter amino acid residue type. Numbers listed apply for CH2. On average, the optimal CH2-TROSY recycle delay was found to be ca. 1 s for Gly-Cα and ca. 0.5 s for side chain CH2.4 Methylene groups in the DNA oligomer. The optimal CH2-TROSY recycle delay for CH2 groups in DNA was 0.8 s.

Application to Sequential Assignment of Nucleic Acids.

The CH2-TROSY enhancement can readily be incorporated in the wide range of commonly used 3D experiments. Here, its benefit is illustrated for the 3D 13C-separated NOESY experiment, applied to DNA.

In nucleic acids, NOE interactions between adjacent nucleotides constitute the mainstay of sequential assignment. For 13C-enriched RNA, the conventional TROSY approach has been shown to improve substantially the 13C resolution of base carbons, benefiting the 3D 13C-separated NOESY experiment for observation of sequential base Hα, Hα′ NOEs.4 However, the shortest distances between sequential nucleotides in a standard duplex helix involve protons in the Cα′ and C′ positions.39 These NOEs are often difficult to analyze because of spectral overlap, especially in the C′ region. Below, we demonstrate that incorporation of the CH2-TROSY element considerably improves spectral resolution, thereby permitting the study of sequential NOEs involving H2/H2′ and H2/H2′ protons.

Table 3 reports the resolution and sensitivity enhancement factors for the C2′ and Cα′ methylene groups in a 19-base-pair DNA fragment,4 when comparing the 2D version of the CH2-TROSY experiment of Figure 2b with that of the analogous methylene-optimized HSQC experiment. Comparison of the Cα′ and C′ methylene groups in a 19-base-pair DNA fragment,4 when comparing the 2D version of the CH2-TROSY experiment of Figure 2b with that of the analogous methylene-optimized HSQC experiment. Comparison of the Cα′ and C′ methylene groups with that of the analogous methylene-optimized HSQC experiment.

References:
regions of the 2D CH$_2$-TROSY and HSQC spectra (Figure 6) confirms a considerable enhancement in spectral resolution, resulting in a nearly 2-fold increase in the number of resolved resonances.

In B-form DNA, the two shortest distances between sequential nucleotides $i$ and $i+1$ involve pairs H$_2''$($i$)/H$_6$/H$_8$($i+1$) and H$_1'$($i$)/H$_8$/H$_8$($i+1$). We demonstrate that the latter interaction can be readily observed when the CH$_2$-TROSY element is incorporated in the 3D 13C-separated NOE experiment. In our implementation (Figure 2b), the TROSY element is placed before the NOE mixing period, such that the enhancement resulting from the 13C Boltzmann magnetization is retained and the relatively well dispersed H$_1'$ and H$_8$/H$_8$ resonances are observed during the detection period, $t_3$. When it is considered that 13C$_2'$ and 13C$_5'$ chemical shifts are well separated from 13C$_1'$,

$^{13}$C$_5'$, and $^{13}$C$_4'$, band-selective adiabatic homonuclear decoupling was used to eliminate the large one-bond $^{1}$J$_{CC}$ couplings ($^{1}$J$_{CC}$ ≈ 40 Hz).$^{40}$

Figure 7 shows small regions of strips taken through the 3D CH$_2$-TROSY-NOESY spectrum of the 19-base-pair DNA oligomer, highlighting some of the NOE connectivities observable for C$_2'$ and C$_5'$ methylene protons. Because of the absence of homonuclear $^{13}$C-$^{13}$C and geminal $^{1}$H-$^{1}$H splittings, as well as the slower transverse relaxation resulting from the CH$_2$-TROSY element, high spectral resolution is obtained in both the indirect $^{13}$C($F_1$) and $^{1}$H($F_2$) dimensions. This, for example, yields resolved correlations for the C$_{22}'$ protons of nucleotide T3, which overlap with T18 in the regular HSQC-NOESY spectrum (data not shown). As expected, sequential NOEs among H$_2''$($T3$)/H$_6$($C4$), H$_2''$($C4$)/H$_6$($T5$), H$_2''$($C4$)/H$_1'$($T3$), and H$_2''$($T5$)/H$_1'$($C4$) are clearly visible and readily permit the sequential assignment to be made. Relatively intense intranucleotide NOE cross-peaks are also visible between H$_2'$ and the
base H6 proton, with a weaker correlation between H5′ and H6 resulting from spin diffusion via H5′ during the relatively long NOE mixing time (250 ms). For this mixing time, intranucleotide correlations between H5′′/H5′ and H5′ are absent and only the H5′′/H1′ sequential connectivity is observed. Therefore, the 3D 13C-separated CH2-TROSY-NOESY experiment offers two separate pathways for sequential assignment, which complement each other in cases of ambiguities resulting from spectral overlap.

**Concluding Remarks**

Detailed analysis of NMR signals involving the abundant methylene protons in macromolecules is frequently hampered by severe spectral crowding and rapid transverse relaxation. This problem provided the impetus behind a powerful but labor-intensive new isotope labeling procedure, known as SAIL,41 where one of each pair of methylene hydrogens is substituted stereospecifically by deuterium. Instead, the CH2-TROSY approach is aimed at the more general case where uniform 13C enrichment, but no selective deuteration, is used. The CH2-TROSY experiment yields considerable enhancement in resolution in both the 1H and 13C dimensions and can offer a modest increase in sensitivity too. As most of the gain in resolution results from dipole–dipole cross-correlation and from removal of the 2JHH splitting, which are both independent of magnetic field strength, the approach is beneficial over the entire range of commonly used magnetic field strengths.

The CH2-TROSY element can be incorporated in a wide variety of NMR experiments, thereby potentially facilitating both resonance assignment and the extraction of structural and dynamic parameters involving methylene groups. The spectral enhancement resulting from the CH2-TROSY element is largest when applied to isolated methylene groups, such as those found in Gly residues. These are often located in turn regions in proteins, whose precise geometry is frequently difficult to identify from the low number of restraints that typically can be extracted for these residues from conventional spectra. When the molecule is weakly aligned relative to the magnetic field, the ability to separately observe any of the methylene 13C–1H multiplet components also permits measurement of all three dipolar couplings for such a group. Besides the important structural information this offers, we currently are also investigating the application of the measurement of three independent dipolar couplings in the plane of the methylene moiety to characterize its dynamic properties.

**Acknowledgment.** We thank F. Delaglio for writing the special processing macro required for the Fourier transform of the 3D TROSY-NOESY and H. Kovacs for help in implementing homonuclear 13C adiabatic decoupling. J.B. is supported by an HFSP fellowship; E.M., by an ARC fellowship; D.C.W., by a PRAT fellowship; and D.L.B., by an NSERC fellowship. This work was supported in part by the Intramural AIDS Targeted Antiviral Program of the Office of the Director of the National Institutes of Health.

**Supporting Information Available:** Four figures showing the following: CH2-TROSY transfer efficiency as a function of transfer delays τ1 and τ2; the relative intensity of spurious peak corresponding to transition C−−1 and C+−1; experimental signal-to-noise as a function of interscan delay in CH2-TROSY; comparison of side chain methylene regions of GB3, recorded with CH2-TROSY and regular HSQC. This material is available free of charge via the Internet at http://pubs.acs.org.

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