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Resolution and sensitivity enhancement of heteronuclear correlation for methylene resonances via ^2H enrichment and decoupling

Diana M. Kushlan and David M. LeMaster*

Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, IL 60208, U.S.A.

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SUMMARY

Monodeuterated methylene positions exhibit substantially superior spectral characteristics in ^1H-^{13}C correlation experiments as compared to diprotio signals. A combination of ^2H decoupling and multiplet editing of HMQC and HSQC experiments provides resolution enhancement for both stereoselective and random fractionally deuterated samples. For HMQC experiments with [2-^2H_R,2-^{13}C]glycine-labeled *E. coli* thioredoxin (11.7 kDa), 3-fold increases in both ^1H and ^{13}C resolution result in a complementary 9-fold enhancement in sensitivity. Owing to a smaller improvement in ^{13}C resolution, the corresponding enhancements for the HSQC experiment are 2-fold less.

To date the vast majority of heteronuclear correlation experiments have made use of single or multiquantum free precession techniques. Several detailed comparisons of the HSQC and HMQC experiments (Norwood et al., 1989,1990; Bax et al., 1990) have discussed effects such as differential relaxation rates for single and multiquantum coherences, ^1H-^1H scalar relaxation and, in the case of the HMQC experiment, ^1H-^1H scalar modulation of the heteronuclear frequency. Little discussion has been presented on the decidedly inferior performance of these experiments for nonequivalent methylene positions as compared to the corresponding methine and methyl resonances. Deuteration techniques offer an exceedingly powerful means of enhancing both the resolution and sensitivity of the methylene resonances for these correlation experiments.

The HMQC spectrum of [2-^{13}C]glycine-labeled *E. coli* thioredoxin is presented in Fig. 1. Eight of the nine glycines give rise to cross peaks involving nonequivalent geminal protons. These cross peaks are substantially broadened in the ^1H dimension by in-phase geminal proton scalar coupling as well as by efficient geminal dipolar relaxation. In the ^{13}C dimension the multiquantum relaxation for typical protein resonances is dominated by ^1H homonuclear dipolar broadening so that

*To whom correspondence should be addressed.

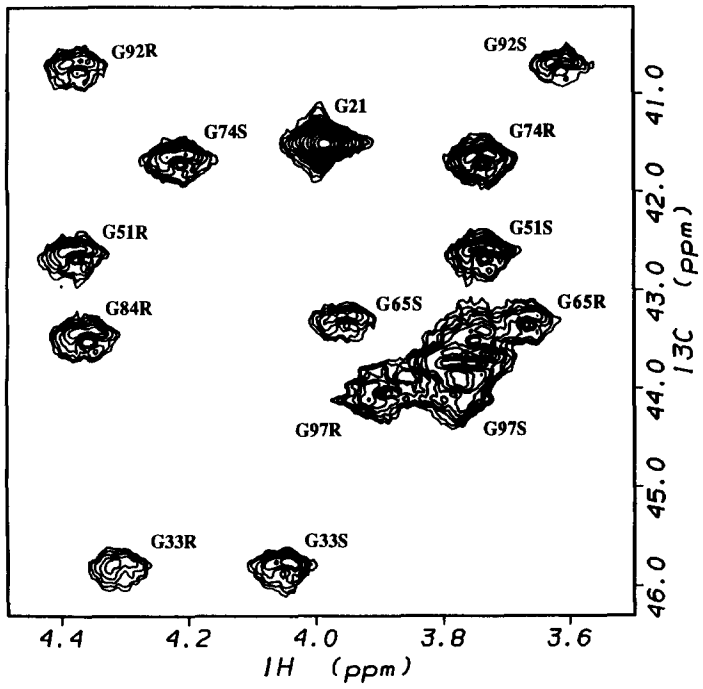

Fig. 1. ^{1}H-^{13}C HMQC spectrum of [2-^{13}C]glycine-labeled *E. coli* thioredoxin at 14.1 T. Sample conditions were 50 mM Na formate, pD 3.9, 25 °C for a 3.5 mM protein concentration. Data was acquired to 132.5 ms in t_1 and 282.6 ms in t_2 with 16 transients per t_1 point. Cosine-bell multiplication was applied to each dimension. These sample, acquisition, and processing conditions are constant for all experiments described except for the 32 transients used in the double refocused HSQC experiment (Fig. 4, panel D).

the relaxation time is approximately that of the ^{1}H T_2 of the directly bonded proton if it were instead attached to ^{12}C (Bax et al., 1990). In addition, the ^{13}C dimension of the HMQC cross peak is broadened and severely distorted by mixed phase modulation from the 15 Hz ^{1}H-^{1}H geminal scalar coupling which is not refocused by the 180° ^{1}H pulse in the middle of the t_1 period. An indication of how much the nonequivalence of the geminal ^{1}H resonances affects the cross peaks is seen by the example of Gly^{21} which exhibits a substantially narrower and more intense cross peak as a result of the degenerate H^{α} resonances.

The ^{1}H-^{1}H geminal scalar and dipolar effects can be eliminated by use of deuterium substitution as illustrated in Fig. 2. Panel A presents an expansion of the most overlapped portion of the HMQC spectrum of Fig. 1. Panel B contains the corresponding HMQC spectrum of [2-$^{2}H_R$, 2-^{13}C]glycine-labeled *E. coli* thioredoxin. Elimination of the ^{1}H-^{1}H geminal scalar and dipolar interactions yields a significant improvement in resolution for the ^{1}H dimension.

In contrast, the resolution in the ^{13}C dimension has not been significantly improved. For some time researchers have recognized the potential utility of ^{2}H substitution as a means of enhancing resolution in ^{13}C protein spectra by elimination of the more efficient ^{1}H-^{13}C dipolar relaxation. In a study of [ε_1-^{2}H,ε_1-^{13}C]histidine-enriched tryptophan synthase α subunit, Browne and co-workers (1973) analyzed the field strength and correlation time dependence of the ^{2}H scalar relaxation contribution to the ^{13}C T_2 relaxation. At lower fields (about 2.4 T), the ^{2}H T_1 relaxation times are submillisecond for correlation times of 5–10 ns. As a result, the ^{2}H-^{13}C scalar interaction is

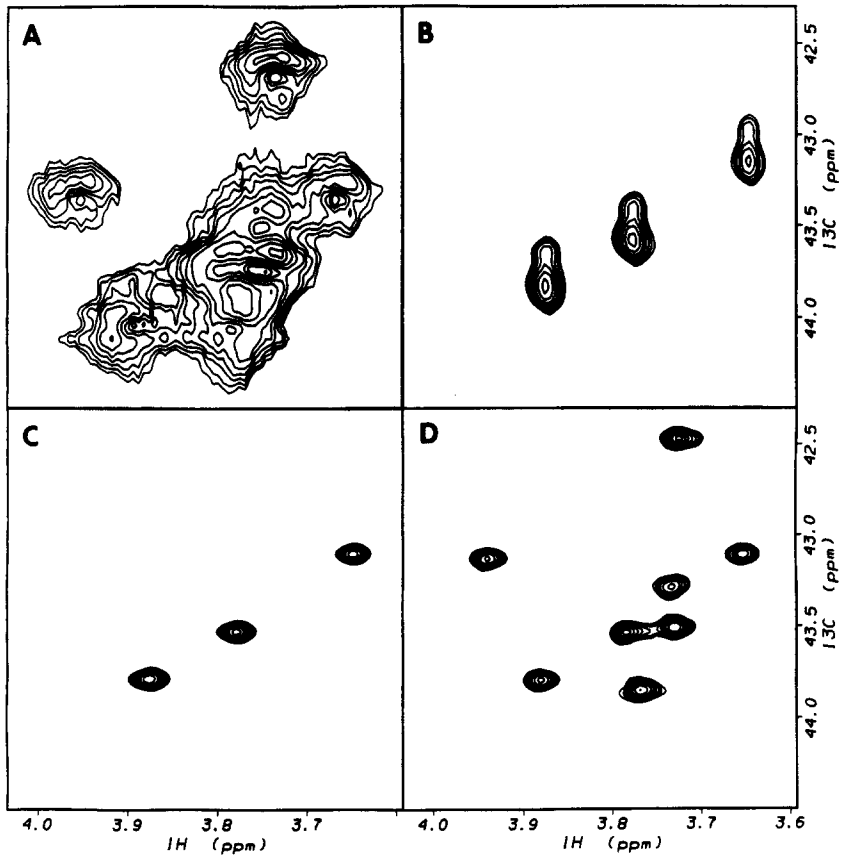

Fig. 2. ^{1}H-^{13}C HMQC spectra as a function of deuteration, ^{2}H decoupling, and multiplet editing. Each panel uses the same multiplicative contouring with the lowest contour set to approximately 25–35% of the peak maxima. (A) Expansion of the HMQC spectrum of [2-^{13}C]glycine-labeled *E. coli* thioredoxin from Fig. 1. (B) Corresponding HMQC spectrum for the [2-$^{2}H_R$,2-^{13}C]glycine-labeled protein. A correlation time-dependent asymmetry in the ^{2}H interaction is observed. [2-^{13}C]Glycine (Cambridge Isotope Lab.) was chirally deuterated by cystathionine γ-synthase catalyzed exchange (Homer, R.J., Kim, M.S. and LeMaster, D.M., manuscript submitted). (C) Collection as for (B) with a WALTZ modulated 3 KHz ^{2}H decoupling field applied from the initial ^{1}H pulse until the start of acquisition. Gated ^{2}H lock system from Bruker Instruments, Inc. (D) DEPT-HMQC spectrum of [50% U-^{2}H,2-^{13}C]glycine-labeled *E. coli* thioredoxin with ^{2}H decoupling. Edit pulse angle set to 45°. Deuteration was obtained by base-catalyzed exchange of [2-^{13}C]glycine (Atkinson et al., 1968; Cable et al., 1987).

partially decoupled. Several studies of deuterated ^{13}C-enriched substrates bound to enzymes have exploited this intrinsic decoupling at lower fields (Jaffe and Markham, 1988; Jaffe et al., 1990; Malthouse and Finucane, 1991). However, since ^{2}H T_1 relaxation becomes less efficient at higher field strengths, in order to benefit from both higher fields and ^{2}H labeling it is necessary to use high-power ^{2}H decoupling combined with gating of the ^{2}H lock channel in order to suppress the ^{2}H scalar interaction efficiently. Recently, Bax and co-workers (Grzesiek et al., 1993) described such a high-power ^{2}H decoupling system and applied it to multidimensional heteronuclear experiments on the random fractionally deuterated, uniformly ^{13}C-enriched calcineurin B protein.

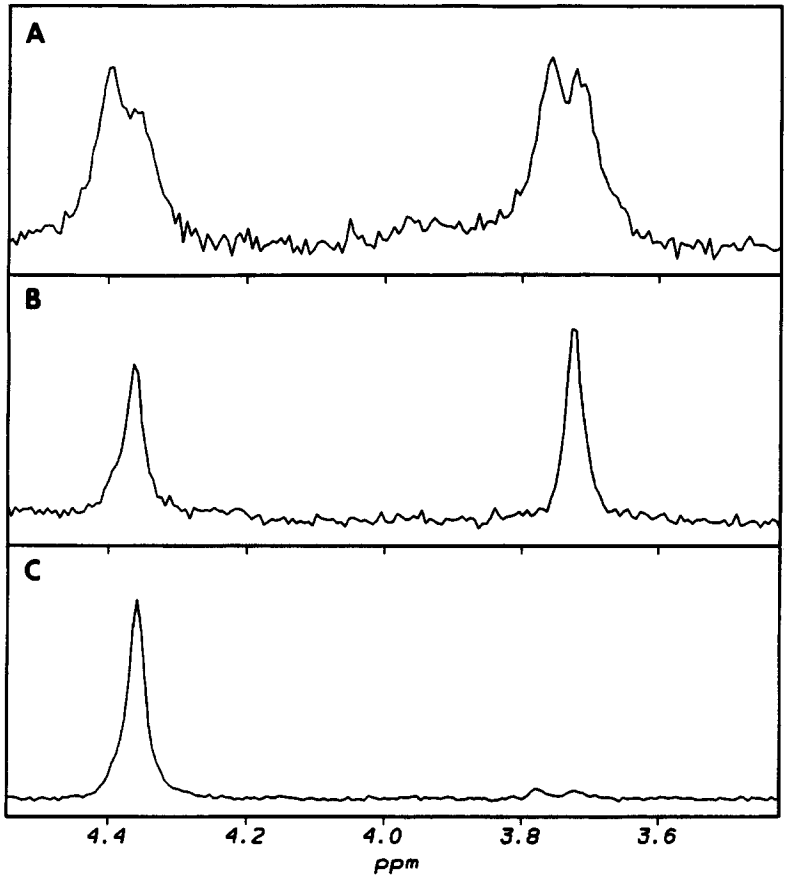

Fig. 3. f_2 slices of ^1H-^{13}C HMQC spectra containing the Gly^{51} C^{α} resonance. (A) HMQC spectrum for [2-^{13}C]glycine-labeled *E. coli* thioredoxin (Fig. 2, panel A). (B) DEPT-HMQC spectrum of the [50% U-^{2}H,2-^{13}C]glycine-labeled sample with ^2H decoupling (Fig. 2, panel D). (C) DEPT-HMQC spectrum of the [2-^2H_R,2-^{13}C]glycine-labeled protein with ^2H decoupling. The intensities of the individual slices are normalized to the maximal peak height.

In each of these earlier ^2H,^{13}C-labeling experiments, the ^{13}C position of interest was effectively perdeuterated. Hence, although the ^{13}C T_2 times can be significantly increased, it is at the sacrifice of a potential ^1H spectral dimension. Heteronuclear correlation of stereoselectively deuterated methylenes has previously been used to obtain resonance assignments (Trimble et al., 1985). Panel C of Fig. 2 demonstrates that, by applying high-power ^2H decoupling for the HMQC spectrum on the [2-^2H_R,2-^{13}C]glycine-labeled sample, it is also possible to gain significant improvement in the resolution of the ^{13}C dimension without sacrificing ^1H detection.

Since stereoselective ^2H labeling is often difficult to achieve, it is desirable to apply these experiments to the more readily accessible random fractionally deuterated protein samples (e.g., Kalbitzer et al., 1985; LeMaster and Richards, 1988; LeMaster, 1990; Grzesiek et al., 1993). However, such an experiment requires the suppression of the signals arising from the diprotio components as can be achieved by spectral editing via a DEPT-HMQC sequence (Kessler et al., 1989). Panel D of Fig. 2 shows the ^2H decoupled DEPT-HMQC spectrum of [50% U-^{2}H,2-

^{13}C]glycine-labeled *E. coli* thioredoxin obtained by using an editing pulse angle of 45° to eliminate the I_2S component.

Comparison of panels A and D (Fig. 2) indicates approximately a 3-fold gain in resolution in both the ^{1}H and ^{13}C dimensions for this 11.7 kDa protein. This enhanced resolution implies a corresponding increase in sensitivity, as illustrated in Fig. 3. Panel A presents the HMQC f_2 slice through the C^α of Gly^{51} of the [2-^{13}C]glycine-labeled sample. The analogous f_2 slice for the ^{2}H decoupled DEPT-HMQC spectrum of the [50% U-^{2}H,2-^{13}C]glycine sample in panel B exhibits a 2-fold greater sensitivity than that of the [2-^{13}C]glycine sample, even though for the [50% U-^{2}H, 2-^{13}C]glycine sample each cross peak arises from only 1/4 of the total molecules. Panel C illustrates the corresponding ^{2}H-decoupled DEPT-HMQC slice for the [^2H_R,2-^{13}C]glycine sample which offers a 7-fold enhancement in sensitivity as compared to the [2-^{13}C]glycine-labeled sample. Indeed, the standard ^{2}H decoupled HMQC experiment with the [2-^2H_R,2-^{13}C]glycine sample is 9-fold more sensitive, indicating a 25% reduction in sensitivity due to the DEPT editing intervals. Since ^{1}H homonuclear dipolar broadening dominates the ^{13}C relaxation in the ^{2}H decoupled HMQC experiments, more extensive deuteration of the protein will further decrease ^{1}H-^{1}H dipole relaxation and hence reduce the ^{13}C linewidth as well.

Although the resolution in the ^{1}H dimension is equivalent, the HSQC experiment offers substantially better ^{13}C resolution than does the HMQC experiment, primarily because of the elimination of the mixed phase ^{1}H-^{1}H scalar modulation. As illustrated by the HSQC spectrum of [2-^{13}C]glycine-labeled *E. coli* thioredoxin in panel A of Fig. 4, the ^{13}C resolution is increased approximately 2-fold over the corresponding HMQC spectrum (Fig. 2, panel A). As expected, the ^{2}H decoupled HSQC spectrum of the [^2H_R,2-^{13}C]glycine sample in panel B demonstrates an improvement in ^{1}H resolution analogous to that for the HMQC experiment while the improvement in ^{13}C resolution is comparatively small.

Comparison of panel C of Fig. 2 and panel B of Fig. 4 indicates a ^{13}C linewidth in the HMQC experiment which is approximately 30% less than that of the HSQC experiment. There is, of course, also a ^{2}H-^{13}C dipolar relaxation contribution to these linewidths. However, due to the $S(S + 1)\gamma^2$ dependence of this interaction, this contribution is expected to be less than 7% for the correlation time of this protein (Kushlan and LeMaster, 1993).

The value of multiplet editing is illustrated in panels C and D. For the ^{2}H-decoupled HSQC spectrum of the [50% U-^{2}H,2-^{13}C]glycine-labeled sample in panel C, each cross peak arising from a monodeuterated ^{13}C position is shadowed by the weaker, more diffuse diprotio cross peak approximately 0.25 ppm downfield in the ^{13}C dimension and 0.01 ppm downfield in the ^{1}H dimension. The distinct spectral frequencies reflect the one-bond ^{2}H-^{13}C and two-bond ^{2}H-^{1}H isotope shifts, respectively (Hansen, 1988). In order to select for the monodeuterated signal, the corresponding ^{2}H-decoupled HSQC spectrum containing a double refocused INEPT sequence (Bax et al., 1990; Kushlan and LeMaster, 1993) is presented in panel D. In this case, since no ^{1}H-^{1}H scalar relaxation effects were anticipated (the amide positions are exchanged in ^2H_2O), a single ^{1}H 180° refocusing pulse during t_1 was used. The delays in the refocusing periods were set to 1.83 ms to suppress the I_2S contributions. Under these conditions the diprotio signal of the [50% U-^{2}H,2-^{13}C]-labeled free amino acid is suppressed approximately 30-fold.

Given the relatively smaller enhancement in ^{13}C resolution, ^{2}H substitution, ^{2}H decoupling and multiplet editing for the HSQC experiment yield enhancements only half as large as those for the HMQC experiment. Hence, there is approximately a 4-fold improvement in resolution and sensi-

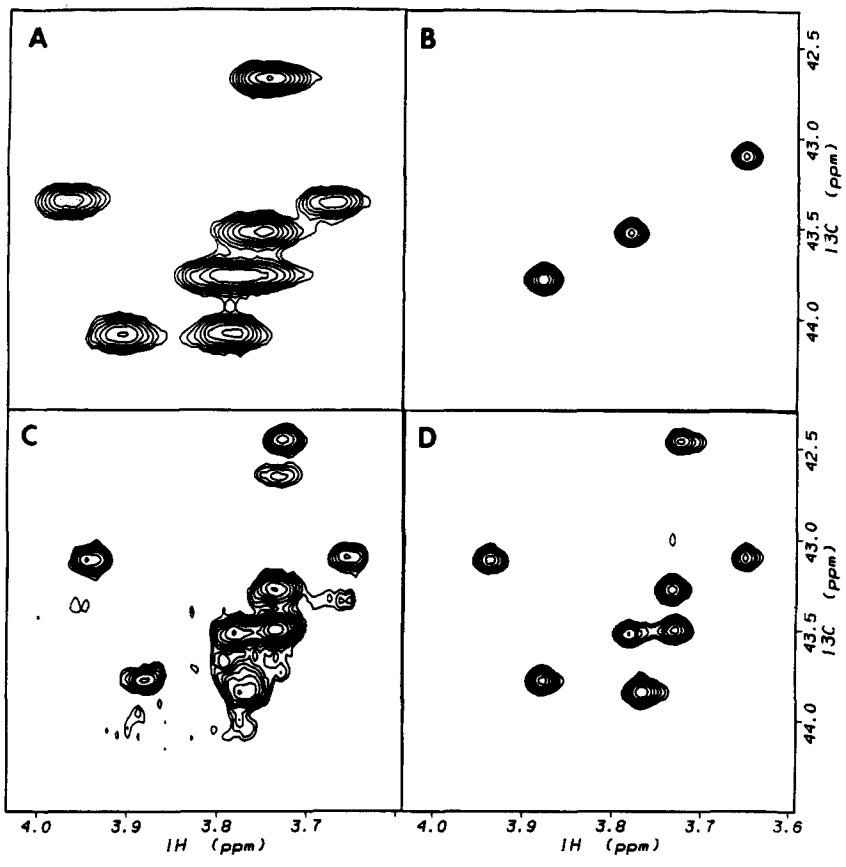

Fig. 4. ^{1}H-^{13}C HSQC spectra as a function of deuteration, ^{2}H decoupling, and multiplet editing. (A) Expansion of a section of the HSQC spectrum of [2-^{13}C]glycine-labeled *E. coli* thioredoxin. (B) Corresponding HSQC spectrum for the [2-$^{2}H_R$,2-^{13}C]glycine-labeled protein with ^{2}H decoupling. (C) HSQC spectrum for the [50% U-^{2}H,2-^{13}C]glycine-labeled protein with ^{2}H decoupling. (D) Double refocused HSQC spectrum for the [50% U-^{2}H,2-^{13}C]glycine-labeled protein with ^{2}H decoupling. The delays in the refocusing periods were set to 1.83 ms.

tivity for the ^{2}H-decoupled HSQC experiment on the [$^{2}H_R$,2-^{13}C]glycine sample as compared to the HSQC experiment on the [2-^{13}C]glycine-labeled protein. Similarly, the sensitivity for the ^{2}H-decoupled refocused HSQC experiment on the [50% U-^{2}H,2-^{13}C]glycine-labeled *E. coli* thioredoxin is approximately equivalent to that for the standard HSQC experiment on the diprotio sample.

A large proportion of 3D and 4D heteronuclear experiments proposed to date utilize a final HMQC or HSQC transfer sequence previous to acquisition. The improved ^{1}H resolution can be readily exploited in the acquisition dimension. However, limitations in digital resolution will often serve to negate the intrinsically narrower ^{13}C linewidths. Conversely, the improved ^{13}C resolution should make lower dimension experiments practical for some applications. For uniformly ^{13}C-enriched samples, constant-time techniques (Bax and Freeman, 1981; Powers et al., 1991) provide for refocusing of ^{13}C-^{13}C couplings to a resolution sufficient to exploit the benefits discussed herein (Vuister and Bax, 1992).

The enhanced resolution in the 2D ^{1}H-^{13}C correlation spectrum is of considerable practical significance for multidimensional experiments in which this correlation represents a terminus of the spin connectivity pathway, as degeneracy in this ^{1}H-^{13}C plane cannot be overcome by simply increasing the dimensionality (Ikura et al., 1990; Grzesiek et al., 1992).

When the proposed experiments are applied to random fractionally deuterated samples, in most cases a mixture of isotopomers will give rise to a set of differential isotope shifts. In the ^{1}H dimension these small isotope shifts (three-bond $^{3}\Delta^{1}H(^{2}H) \sim 0.008$ ppm (Hansen, 1988)) have a rather small effect on resolution as previously noted in ^{1}H homonuclear experiments (LeMaster and Richards, 1988; LeMaster, 1990). The differential isotope shift effects resulting from a mixture of ^{2}H isotopomers present a comparatively greater problem for ^{13}C resolution due to two different factors. At 14.1 T the $^{2}\Delta^{13}C(^{2}H)$ and $^{3}\Delta^{13}C(^{2}H)$ isotope shifts are approximately 12–15 Hz and 3–4 Hz, respectively (Majerski et al., 1985; Hansen, 1988), as large or larger than the corresponding ^{1}H-^{13}C scalar couplings. Furthermore, unlike the passive ^{1}H-^{1}H scalar couplings in the ^{1}H dimension, the long-range ^{1}H-^{13}C scalar couplings are refocused in the standard heteronuclear correlation experiments and thus do not contribute to the observed linewidth. Such an additional broadening effect will be comparable to the ^{13}C linewidths observed for the monodeuterated glycine resonances of *E. coli* thioredoxin. However, although the differential isotope shift effects are molecular weight independent, in the HMQC experiment the benefit of ^{13}C resolution resulting from suppression of the ^{1}H-^{1}H dipolar relaxation via random fractional deuteration will increase for larger protein systems.

The largest proteins for which extensive resonance assignment studies have been carried out to date have expected correlation times of 15–20 ns compared to the 8 ns observed for *E. coli* thioredoxin under these conditions (Kushlan and LeMaster, 1993). The data presented here strongly indicates that even in these cases substantial enhancement in resolution and sensitivity, particularly for the HMQC experiment, can be anticipated from the use of ^{2}H labeling and decoupling techniques.

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