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Improved ^{13}C-Resolved HSQC-NOESY Spectra in H_2O, Using Pulsed Field Gradients

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Accurate structure determination of large proteins ($M_r >$ 15 kDa) using multidimensional NMR ultimately relies on the number of $^1H-^1H$ NOE intensities that can be unequivocally assigned and reliably quantified (*1*). In conventional homonuclear 2D NOESY spectra, severe overlap of resonances prevents assignment as well as measurement of a large number of NOE cross peaks. In recent years, ^{13}G- and ^{15}N-resolved 3D and 4D NOESY experiments of uniformly isotope-labeled proteins have substantially alleviated these problems (*2, 3*). In these experiments, an NOE between two protons is dispersed into a third or fourth dimension on the basis of the frequency of an associated heteronucleus. In a 3D ^{13}C-resolved HSQC-, HMQC-, or HSMQC-NOESY spectrum (*4*), NOEs from an alpha proton, for example, to the side chain are edited by the $^{13}C_\alpha$ chemical shift in the third dimension. Apart from resolving NOEs between aliphatic protons, ^{13}C-resolved NOESY spectra present an additional advantage when recorded with H_2O as solvent, where these spectra also yield a significant number of aliphatic–amide proton NOEs, edited by the aliphatic ^{13}C frequencies. These cross peaks not only provide valuable sequential, medium- and long-range NOE constraints for structure determination, but also aid considerably in the resonance assignment process. Therefore, it would indeed be most desirable to be able to record high-quality ^{13}C-resolved NOESY spectra of proteins in H_2O.

The single problem that has to date plagued experiments performed in H_2O has been, of course, suppression of the solvent signal. Although a large number of water-suppression schemes have appeared in the literature, presaturation, with or without the addition of spin-lock trim pulses (*5*), remains the most commonly used method. The use of presaturation invariably results in the bleaching out of H_α/H_β resonances lying close to the H_2O resonance (usually ±0.15 ppm) and also leads to undesirable cross-relaxation effects which are very efficient in large proteins. Another disadvantage of presaturation is saturation transfer between H_2O and NH protons, especially in the high-pH samples that are commonly used at present. These effects combine to degrade spectral quality, reduce sensitivity, and introduce unquantifiable contributions to the NOE cross-peak intensities. Although techniques such as SCUBA (*6*) have been proposed for partial recovery of some bleached-out resonances, it is certainly most desirable to be able to perform the experiment with no perturbation to the sample magnetization before the pulse sequence, in order to retain full sensitivity and obtain fully quantifiable data.

Recently, pulsed field gradients (PFG) have been introduced as effective tools for solvent suppression. A conceptually elegant application uses PFGs for coherence-pathway selection, which completely eliminates the need for presaturation or even phase cycling (*7–13*). This approach, however, suffers from a sensitivity loss by a factor of $\sqrt{2}$ because only one-half of the total magnetization is refocused by the gradients. This is an undesirable feature, given the low sample concentrations that are normally available.

The other, more mundane approach is to use PFGs as coherence spoilers for artifact suppression and/or "heavy-duty" *z* and *zz* filters (*14–17*). While this approach cannot eliminate phase cycling completely, it has the advantage of combining full sensitivity with efficient solvent suppression. The essential idea behind the *z* and *zz* filter approach is to convert relevant coherences into *zz* spin order or *z* magnetization while the transverse solvent and other undesirable coherences are dephased by the gradient pulses. Brühwiler and Wagner (*18*), Zuiderweg (*19*), and, more recently, Hurd and co-workers (*16*) have demonstrated how PFGs can be effectively used as *z* and *zz* filters. Sklenář *et al.* (*17*) have used *zz* filtration in an ^{15}N-resolved NOESY-HSQC experiment, in conjunction with a WATERGATE (*20*) sequence, where the water resonance is defocused using a pair of identical gradient pulses sandwiching a selective 180° pulse on the amide protons. While this technique is very well suited for ^{15}N-resolved spectra, in a ^{13}C-resolved HSQC-NOESY experiment the entire proton spectrum is of interest and, therefore, selective 180° pulses cannot be applied. In this Communication, we have chosen to demonstrate the use of the *z* and *zz* filter "units," proposed by Hurd and co-workers, in a ^{13}C-resolved HSQC-NOESY experiment. Although the

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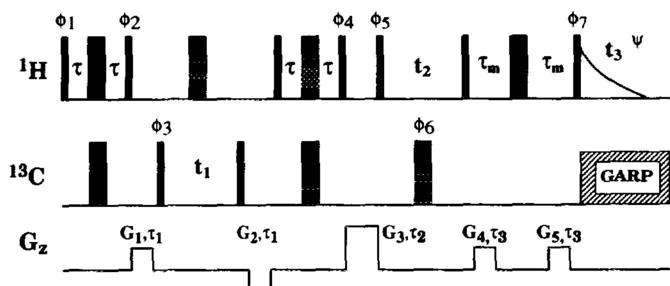

FIG. 1. Pulse sequence for the gradient-enhanced ^{13}C-resolved HSQC-NOESY experiment. Phase cycles are as follows: ϕ_1 = 4(x), 4(−x); ϕ_2 = 2(y),2(−y); ϕ_3 = x,−x; ϕ_4 = y; ϕ_5 = x; ϕ_6 = 8(x),8(−x); ϕ_7 = 4(x), 4(y), 4(−x), 4(−y); ψ(receiver) = x, −x, −x, x, −y, y, y, −y, −x, x, x, −x, y, −y, −y, y. The delay τ was set to 1.60 ms and the total mixing time ($2\tau_m + 2\tau_3$) was 80 ms. All PFGs were rectangular. Gradient strengths and durations were as follows: G_1, G_2, G_4, G_5 = 6 G/cm; G_3 = 18 G/cm; τ_1 = 2 ms; τ_2, τ_3 = 5 ms. Quadrature detection in t_1 and t_2 was performed by incrementing the phases ϕ_3 and ϕ_5 using the States–TPPI method (21). ^{13}C decoupling during acquisition was performed using the GARP (22) sequence. As the sample was also ^{15}N labeled, it was necessary to perform ^{15}N decoupling during acquisition, for which a WALTZ-16 (23) sequence was used.

application is quite straightforward, our aim here is to emphasize the outstanding quality of spectra in H_2O that can be obtained without any degree of presaturation or trim pulses and without distortion of the spectral excitation window.

The pulse sequence for the gradient-enhanced ^{13}C-resolved HSQC-NOESY spectrum is shown in Fig. 1. The gradients G_1 and G_2 are zz filters whereas G_3 is a z filter. G_4 dephases any residual transverse H_2O magnetization during the NOESY period. G_5 is optional and defocuses any additional transverse H_2O magnetization created by imperfections in the 180° pulse in the center of the NOESY period. We found marginal, but notable, improvement in water suppression in our experiment. We did not use any selective-excitation–dephase sequences at the beginning of the sequence as reported by Hurd and co-workers (16), thus ensuring that the protein magnetization is totally undisturbed prior to the pulse sequence. Even so, excellent water suppression was achieved per scan.

The experiment was performed using a 1.5 mM sample of T4 lysozyme (18.7 kDa) uniformly labeled (>95%) with ^{13}C and ^{15}N, dissolved in 90% H_2O/10% D_2O solution at 30°C. The spectrum was recorded on a Bruker AMX 500 spectrometer equipped with a self-shielded triple-resonance z gradient probe. The PFGs were generated using a Bruker GRASP unit. The NOESY mixing time used (80 ms) was well below the optimum for cross-peak intensities in T4 lysozyme. Even so, the good quality of the data is apparent

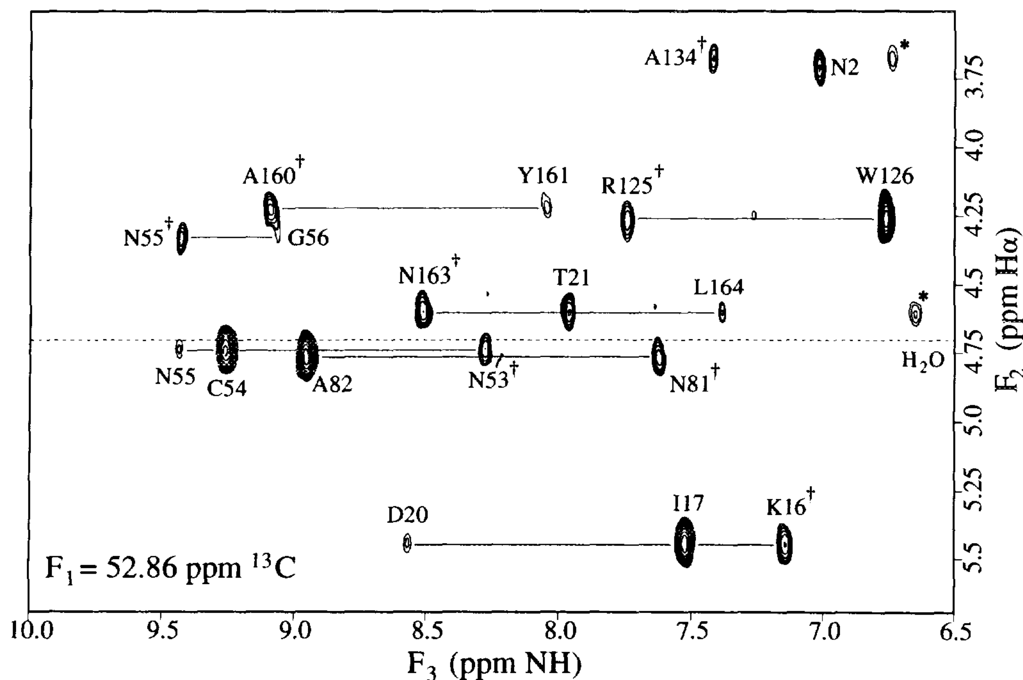

FIG. 2. A $^{13}C_\alpha$ plane showing Hα–NH cross peaks from the ^{13}C-resolved HSQC-NOESY spectrum of T4 lysozyme. Acquisition parameters (t_1,t_2,t_3) were as follows: carrier (ppm), 41.60, 4.75, and 4.75; acquisition time (ms), 21.1, 25.9, and 170.0; data points (complex), 60, 148, and 1024. The ^{13}C dimension was folded. Sixteen scans were acquired per (t_1,t_2) increment. A relaxation delay of 1.0 s was used between scans. The total duration of the experiment was approximately 114 hours. Data were processed on a Silicon Graphics workstation using Felix 2.0 and Felix 2.05 (Hare Research, Inc.), augmented with several in-house routines. The final matrix dimensions and digital resolution (Hz/pt) in F_1, F_2, and F_3 were 256 (22.2), 256 (5.6), and 1024 (5.9).

from Fig. 2, which shows the $H_\alpha(F_2)$–$NH(F_3)$ NOESY cross peaks from a $^{13}C_\alpha(F_1)$ plane in the spectrum. Self-NOEs are labeled with a "†" and sequential connectivities are drawn with solid lines. The dashed line represents the water resonance frequency. It can immediately be noted that NOEs from the H_α resonances of N53 (H_α = 4.74 ppm) and N81 (H_α = 4.75 ppm) which are almost at the water frequency are undisturbed. In fact, all H_α protons in the range 4.5–4.9 ppm (12% of T4 lysozyme residues), which were almost always bleached out or too weak in presaturated versions of the experiment, are present in this spectrum. The peaks marked with an "*" are NOEs yet unassigned to side chain amides or aromatic ring protons. The self-NOE from D_2O and the sequential NOE from A134 to K135 are too weak to be seen on this plane. The relative intensities of the self versus sequential NOEs are in accordance with our present knowledge of the secondary structure of T4 lysozyme (*1, 24*).

In conclusion, we have presented an application of gradient-enhanced ^{13}C-resolved HSQC-NOESY experiment which retains full sensitivity, achieves high water suppression, and provides excellent-quality spectra which can be quantitatively used for structure determination. Although, in principle, an HMQC-NOESY spectrum has higher intrinsic sensitivity owing to the smaller number of pulses involved, there is little scope for introducing *z* and *zz* filtration, which forces the use of at least some presaturation and/or jump-return techniques, which largely offset the sensitivity gain and spectral quality that may be obtained in the *z*- and *zz*-filtered HSQC-NOESY spectrum.

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REFERENCES

1. K. Wüthrich, "NMR of Proteins and Nucleic Acids," Wiley, New York, 1986.
2. S. W. Fesik and E. R. P. Zuiderweg, *Q. Rev. Biophys.* **23,** 97 (1990).
3. G. M. Clore and A. M. Gronenborn, *Prog. NMR Spectrosc.* **23,** 43 (1991).
4. E. R. P. Zuiderweg, L. P. Mcintosh, F. W. Dahlquist, and S. W. Fesik, *J. Magn. Reson.* **86,** 210 (1990).
5. B. A. Messerle, G. Wider, G. Otting, C. Weber, and K. Wüthrich, *J. Magn. Reson.* **85,** 608 (1989).
6. S. C. Brown, P. L. Weber, and L. Müller, *J. Magn. Reson.* **77,** 166 (1988).
7. R. E. Hurd, *J. Magn. Reson.* **87,** 422 (1990).
8. R. E. Hurd and B. K. John, *J. Magn. Reson.* **92,** 658 (1991).
9. G. W. Vuister, R. Boelens, R. Kaptein, M. Burgering, and P. C. M. van Zijl, *J. Am. Chem. Soc.* **113,** 9688 (1991).
10. A. L. Davis, J. Keeler, E. Laue, and D. Moskau, *J. Magn. Reson.* **98,** 207 (1992).
11. J. R. Tolman, J. Chung, and J. H. Prestegard, *J. Magn. Reson.* **98,** 467 (1992).
12. J. Ruiz-Cabello, G. W. Vuister, C. T. W. Moonen, P. van Gelderen, J. S. Cohen, and P. C. M. van Zijl, *J. Magn. Reson.* **100,** 282 (1992).
13. A. Ross, M. Czisch, C. Cieslar, and T. A. Holak, *J. Biomol. NMR* **3,** 215 (1993).
14. A. Bax and S. S. Pochapsky, *J. Magn. Reson.* **99,** 638 (1992).
15. G. W. Vuister, G. M. Clore, A. M. Gronenborn, R. Powers, D. S. Garrett, R. Tschudin, and A. Bax, *J. Magn. Reson. B* **101,** 210 (1993).
16. B. K. John, D. Plant, and R. E. Hurd, *J. Magn. Reson. A* **101,** 113 (1992).
17. V. Sklenář, M. Piotto, R. Leppik, and V. Saudek, *J. Magn. Reson. A* **102,** 241 (1993).
18. D. Brühwiler and G. Wagner, *J. Magn. Reson.* **69,** 546 (1986).
19. E. R. P. Zuiderweg, *J. Magn. Reson.* **71,** 283 (1987).
20. M. Piotto, V. Saudek, and V. Sklenář, *J. Biomol. NMR* **2,** 661 (1992).
21. D. Marion, M. Ikura, R. Tschudin, and A. Bax, *J. Magn. Reson.* **85,** 393 (1989).
22. A. J. Shaka, P. B. Barker, and R. Freeman, *J. Magn. Reson.* **64,** 547 (1985).
23. A. J. Shaka, J. Keeler, T. Frenkiel, and R. Freeman, *J. Magn. Reson.* **52,** 335 (1983).
24. L. H. Weaver and B. W. Matthews, *J. Mol. Biol.* **193,** 189 (1987).