JOURNAL OF MAGNETIC RESONANCE 95, 636–641 (1991)

An Alternative 3D NMR Technique for Correlating Backbone ^{15}N with Side Chain Hβ Resonances in Larger Proteins

SHARON J. ARCHER,* MITSUHIKO IKURA,† DENNIS A. TORCHIA,* AND AD BAX†

*Bone Research Branch, National Institute of Dental Research, and †Laboratory of Chemical Physics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

Received August 9, 1991

Values of ^{15}N–^{1}H spin–spin coupling constants can be measured in a quantitative manner by recording ^{1}H–^{1}H TOCSY/HOHAHA or NOESY spectra of a ^{15}N-enriched protein in the absence of ^{15}N decoupling (*1*). Although this technique is elegant and potentially very powerful, the absence of ^{15}N decoupling halves the signal-to-noise ratio and may make the small displacements of broad resonances which contain the long-range J_{NH} information difficult to measure. Moreover, for larger proteins the TOCSY/HOHAHA cross peaks frequently are too weak to be detected at all.

As recently shown by Chary *et al.* (*2*), measurement of cross-peak intensity in a 3D spectrum that correlates amide proton and ^{15}N chemical shifts with those of the intraresidue Hβ resonances provides qualitative information on the size of the three-bond ^{15}N–Hβ J coupling. This yields information about the side-chain torsion angle χ_1 and aids with the stereospecific assignment of nonequivalent methylene protons. In their experiment, net magnetization is transferred from the amide proton via its ^{15}N to the side-chain Hβ protons, which are detected during the acquisition period, t_3, of the 3D experiment. At the beginning of t_3, there is no net magnetization and only after rephasing with respect to the ^{15}N nucleus takes place can the magnetization be detected. In larger proteins, the line widths of Hβ methylene protons are among the largest in the protein because of their strong geminal dipolar interactions, which are superimposed on large and often unresolvable geminal and vicinal ^{1}H–^{1}H J couplings. Here we propose a simple modification of this experiment that removes this dependence on the Hβ linewidth and which therefore can be applied to proteins of a substantially larger size. Instead of using net magnetization transfer, the new technique relies on the "out-and-back" magnetization-transfer approach previously used in a series of other 3D triple-resonance techniques (*3–6*).

The pulse scheme for the new experiment is sketched in Fig. 1A. Because the main use of this experiment is to correlate amide proton (HN) and nitrogen resonances with side-chain Hβ proton signals, we name the experiment HNHB. Briefly, the magnetization transfer goes as follows: Magnetization is transferred from the amide proton to the amide nitrogen using an INEPT transfer. During the subsequent evolution period, t_1, the antiphase magnetization evolves with the chemical shift of the ^{15}N

0022-2364/91 $3.00
Copyright © 1991 by Academic Press, Inc.
All rights of reproduction in any form reserved.

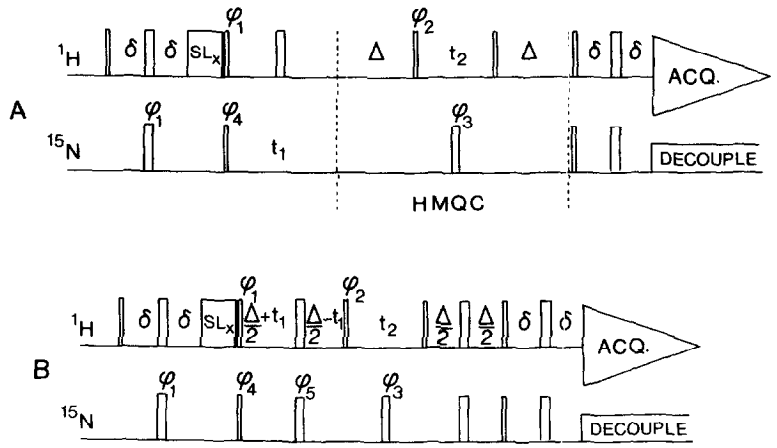

FIG. 1. Pulse schemes for the HNHB experiment. (A) Regular pulse scheme, where the t_1 evolution period precedes the ^{15}N–Hβ heteronuclear multiple-quantum correlation (HMQC) fragment (between vertical dashed lines). (B) Constant-time version of the HNHB experiment, where the t_1 evolution period has been integrated in the first Δ delay of the ^{15}N–Hβ HMQC. Narrow and wide pulses represent 90° and 180° flip angles. Unless marked, all pulses are applied along the x axis. The phase cycling used is as follows. Scheme A: $\phi_1 = y, -y$; $\phi_2 = 2(x), 2(-x)$; $\phi_3 = 4(x), 4(y), 4(-x), 4(-y)$; Acq. $= x, 2(-x), x, -x, 2(x), -x$. Scheme B: As in scheme (A) with $\phi_5 = 16(x), 16(-x)$. Quadrature detection in the t_1 dimension is performed in a States–TPPI manner (*18*), with the phase ϕ_4 altered for the t_1 dimension, and ϕ_2 for the t_2 dimension. The spin-lock pulse (SL_x) with a duration of 1 ms serves to lock the antiphase ^{15}N magnetization, while the water magnetization, perpendicular to the x axis, is being destroyed (*19*). In addition, in our experiments presaturation of the H_2O resonance with a very weak RF field of ~10 Hz was used during the delay time between scans (800 ms) to further attenuate the H_2O resonance. The delay τ is set slightly shorter than $1/(4J_{NH})$, 2.25 ms in practice, and Δ is set to 38 ms.

nucleus. Because a ^{1}H 180° pulse is applied at the midpoint of t_1, ^{15}N magnetization remains antiphase with respect to its attached proton at the end of t_1. The next step, between the dotted lines in Fig. 1A, represents a HMQC correlation (*7–9*) between the ^{15}N nucleus and other protons that have a long-range coupling with the ^{15}N. Note that the correlation to the directly attached amide proton is minimized by setting the delay, Δ, to an odd multiple of $1/(2J_{NH})$ (38 ms in our experiments), ensuring that the ^{15}N transverse magnetization is in phase with respect to its directly attached amide proton when the $90^\circ_{\phi 2}$ ^{1}H pulse is applied. Finally, the transverse ^{15}N magnetization, now modulated by the chemical shifts of its long-range-coupled protons, is transferred back to its directly attached proton by a reverse INEPT transfer and the ^{1}H signal is detected during t_3 in the presence of broadband ^{15}N decoupling.

The amplitude of a correlation between the amide proton, H, its ^{15}N nucleus, N, and a proton, B, long-range coupled to N, is proportional to

$$\sin^2(\pi J_{NB}\Delta)\sin^2(\pi J_{NH}\Delta)\prod_k \cos^2(\pi J_{Nk}\Delta)\exp(-2\Delta/T_{2N}), \qquad [1]$$

where the product extends over all protons, k, other than B that have a long-range coupling with N, and T_{2N} is the ^{15}N transverse relaxation time in the presence of ^{1}H coupling, which is ~30% faster than that in the presence of ^{1}H decoupling (*10, 11*).

Note that the integrated cross-peak intensity is independent of the linewidth of the long-range-coupled proton B, or of its $^1H-^1H$ multiplet structure. The success of the experiment depends primarily on the size of the long-range coupling of interest, J_{NB}, relative to the ^{15}N linewidth ($1/\pi T_{2N}$).

Although the interresidue $^3J_{NH\alpha}$ values do not obey a simple Karplus relationship (*12, 13*), intraresidue $^3J_{NH\beta}$ couplings can be related to the dihedral angle χ_1 in the regular manner, with an absolute value for the trans coupling of about 5 Hz (*12, 14*). The two-bond and three-bond $J_{NH\alpha}$ couplings are generally smaller than this. For a duration of the delay Δ (Fig. 1) equal to 38 ms, the passive couplings J_{Nk} in expression [1] therefore do not significantly attenuate signal intensity, and the observed correlation to a proton, B, is approximately proportional to J_{NB}^2. Note, however, that the intensity also depends strongly on the transverse relaxation rate of the amide ^{15}N resonance and on the degree of attenuation of the amide proton by presaturation of the H_2O resonance. Therefore, strictly speaking one can only compare relative intensities of correlations observed for a given amide, unless both the T_{2N} value (in the ^1H-coupled mode) and the attenuation resulting from presaturation are measured and taken into account.

The HNHB experiment has been applied to the proteins calmodulin ($M_r \sim 16.7$ kDa) and TGF-β1, a covalently cross-linked homodimer of ~25 kDa. Both proteins were uniformly (>95%) enriched with ^{15}N and both samples were dissolved in 95%/5% H_2O/D_2O at concentrations of 1.5 and 0.9 m*M* (dimer), respectively, and measuring times were about three days for each of the 3D spectra. Experiments were carried out on an unmodified Bruker AMX-600 spectrometer, either at 35°C (calmodulin) or 47°C (TGF-β1), T_{2N} values, as measured from the ^{15}N linewidths in an Overbodenhausen (HSQC) spectrum, are ~70 ms for calmodulin and ~45 ms for TGF-β1, although significant variation of T_{2N} is observed within each protein.

Figure 2 shows an (F_2, F_3) slice taken from the 3D HNHB spectrum of calmodulin, taken at a ^{15}N chemical shift of 120.1 ppm. As expected, for most residues with nonequivalent Hβ protons only a single correlation is observed. However, for a number of residues (e.g., Leu-48 and Glu-114) both Hβ protons are visible, albeit with different intensity. This can be caused either by rapid rotation about the χ_1 angle, resulting in an averaging of multiple side-chain conformers, or by a skewed rotamer in which one of the Hβ protons gets close to a syn orientation relative to the ^{15}N. In this particular slice, relatively few correlations to Hα protons are observed. However, in the entire 3D spectrum, 28 intraresidue and 24 interresidue correlations to Hα protons were identified.

Despite its substantial size, calmodulin has relatively narrow ^{15}N lines, caused in part by flexibility of the "central helix" which connects the two domains (Barbato, to be published). The protein TGF-β1 presents a more challenging case. It is a symmetric dimer of yet unknown structure, covalently linked by disulfide bridges. Although the molecular weight of the dimer is relatively large by NMR standards, the signals of each 112-residue monomer are equivalent, resulting in a relatively well-resolved $^1H-^{15}N$ correlation spectrum. The principal use for the HNHB method with this protein at the initial stage of our NMR study is not for obtaining stereospecific assignments, but rather for linking Hβ protons with the intraresidue amide proton. For this protein very few such correlations could be obtained from the 2D and 3D HOHAHA spectra.

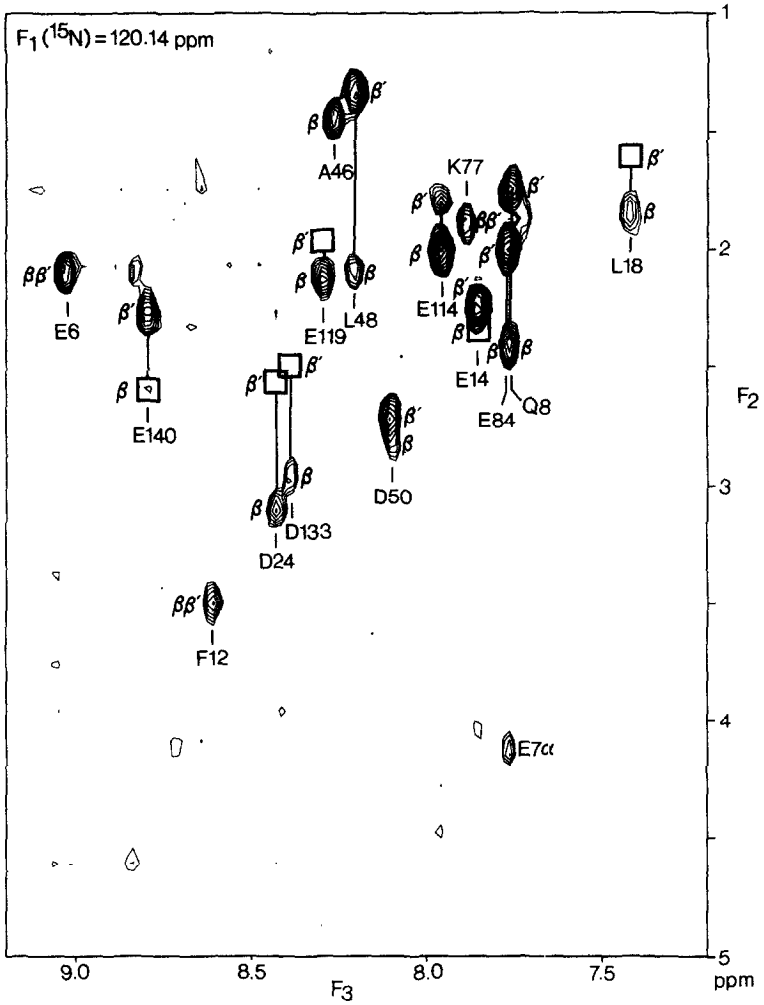

FIG. 2. Region of a ^1H-^1H slice taken from the 3D HNHB spectrum of calmodulin, complexed with Ca^{2+}. The 3D spectrum has been recorded with the scheme of Fig. 1A and results from a (32 complex) × (64 complex) × (256 complex) data matrix with acquisition times of 19.2 ms (^{15}N, t_1), 12.8 ms (Hβ, t_2), and 51.2 ms (HN, t_3). The total measuring time was about 67 hours.

Figure 3 shows a slice from the 3D HNHB spectrum, demonstrating that indeed this method is applicable to a protein of 224 residues. As expected, the signal-to-noise ratio is significantly lower than that of the calmodulin spectrum, but nevertheless, 77 correlations to Hβ protons were identified in the 3D spectrum. Only 10 correlations to Hα protons were observed.

The method shown here uses a relatively small number of pulses. It can be improved, albeit at the cost of three extra 180° pulses, by making it of the "constant-time" variety (*15–17*). This is accomplished by overlaying the first Δ delay with the constant-time ^{15}N evolution period, in a manner as sketched in Fig. 1B. In the constant-time HNHB

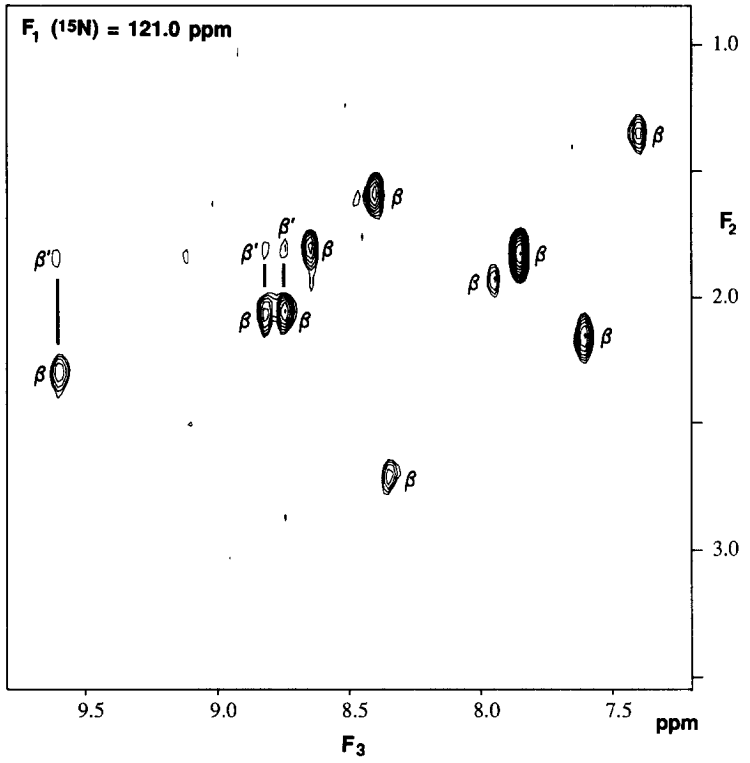

FIG. 3. HN–Hβ region of an F_2–F_3 slice taken from the 3D HNHB spectrum of TGF-β1, taken at an F_1 (^{15}N) frequency of 121 ppm. All parameters except for the relaxation delay time between scans (0.9 s) and the total measuring time (74 h) were as reported in the legend to Fig. 2.

experiment there is no signal decay caused by transverse relaxation in the t_1 dimension, possibly further increasing the size of proteins to which the HNHB experiment can be applied.

ACKNOWLEDGMENTS

We thank Michael Sporn for providing us with the sample of ^{15}N-enriched TGF-β. This work was supported by the AIDS directed anti-viral program of the Office of the Director of the National Institutes of Health.

REFERENCES

1. G. T. MONTELIONE, M. E. WINKLER, P. RAUENBUEHLER, AND G. WAGNER, *J. Magn. Reson.* **82,** 198 (1989).
2. K. V. R. CHARY, G. OTTING, AND K. WÜTHRICH, *J. Magn. Reson.* **93,** 218 (1991).
3. M. IKURA, L. E. KAY, AND A. BAX, *Biochemistry* **29,** 4659 (1990).
4. L. E. KAY, M. IKURA, R. TSCHUDIN, AND A. BAX, *J. Magn. Reson.* **89,** 496 (1990).
5. G. WAGNER, P. SCHMIEDER, AND V. THANABAL, *J. Magn. Reson.* **93,** 436 (1991).
6. R. POWERS, A. M. GRONENBORN, G. M. CLORE, AND A. BAX, *J. Magn. Reson.* **94,** 209 (1991).
7. L. MUELLER, *J. Am. Chem. Soc.* **101,** 4481 (1979).
8. M. R. BENDALL, D. T. PEGG, AND D. M. DODDRELL, *J. Magn. Reson.* **52,** 81 (1983).

9. A. BAX, R. H. GRIFFEY, AND B. L. HAWKINS, *J. Magn. Reson.* **55**, 301 (1983).
10. A. BAX, M. IKURA, L. E. KAY, D. A. TORCHIA, AND R. TSCHUDIN, *J. Magn. Reson.* **86**, 304 (1990).
11. L. E. KAY, L. K. NICHOLSON, F. DELAGLIO, A. BAX, AND D. A. TORCHIA, submitted for publication.
12. A. DEMARCO, M. LLINAS, AND K. WÜTHRICH, *Biopolymers* **17**, 2727 (1978).
13. K. D. KOPPLE, A. ASHAN, AND M. BARFIELD, *Tetrahedron Lett.* **38**, 3519 (1978).
14. V. F. BYSTROV, *Prog. NMR Spectrosc.* **10**, 41 (1976).
15. A. BAX, A. F. MEHLKOPF, AND J. SMIDT, *J. Magn. Reson.* **35**, 167 (1979).
16. O. W. SØRENSEN, *J. Magn. Reson.* **90**, 433 (1990).
17. M. IKURA, L. E. KAY, AND A. BAX, *J. Biomol. NMR*, in press.
18. D. MARION, M. IKURA, R. TSCHUDIN, AND A. BAX, *J. Magn. Reson.* **85**, 393 (1989).
19. B. A. MESSERLE, G. WIDER, G. OTTING, C. WEBER, AND K. WÜTHRICH, *J. Magn. Reson.* **85**, 608 (1989).