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# *J*-Multiplied HSQC (MJ-HSQC): A New Method for Measuring $^3J(H_NH_\alpha)$ Couplings in $^{15}$N-labeled Proteins

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**A new method for the measurement of homonuclear $^3J(H_NH_\alpha)$ coupling constants in $^{15}$N-labeled small proteins is described. The method is based on a modified sensitivity enhanced HSQC experiment, where the $^3J(H_NH_\alpha)$ couplings are multiplied in the $f_1$-dimension. The *J*-multiplication of homonuclear $^3J(H_NH_\alpha)$ couplings is based on simultaneous incrementation of $^{15}$N chemical shift and homonuclear coupling evolution periods. The time increment for the homonuclear coupling evolution period is chosen to be a suitable multiple ($2N \times t_1$) of the corresponding increment for $^{15}$N-shift evolution. This results in the splitting of the HSQC correlation in the $f_1$-dimension by $2N \times {}^3J(H_NH_\alpha)$. Because the pulse sequence has good sensitivity and water suppression properties, it is particularly useful for natural abundance samples.**



*Key Words:* **NMR; HSQC; coupling constant; ubiquitin.**

## INTRODUCTION

The $\phi$-angle is an important parameter for the calculation of protein structures from NMR data. In literature, several methods have been proposed for the determination of $^3J(H_NH_\alpha)$ coupling constants, which can be related to the $\phi$-angle using an appropriately parametrized Karplus equation (*1, 2*). However, accurate measurement of homonuclear coupling constants in proteins is often problematic as the linewidths are large and relaxation times short.

In principle, the antiphase splitting in a COSY spectrum can be measured and fitted with observed (or assumed) linewidths (*3–5*). Also, *J*-modulated, HSQC based methods have been suggested (*6–8*), as well as methods that are based on triple resonance techniques with $^{15}$N/$^{13}$C-labeled proteins (*9, 10*). The quantitative *J*-correlation method (*8, 11*) relies on the measurement of intensity differences between diagonal and cross peaks in a 3D HNHA spectrum of $^{15}$N-labeled proteins. The triple resonance-based methods, as well as the HNHA, are relatively robust, but problems may be encountered with larger proteins. In the triple resonance-based experiments, the relaxation during the evolution period may interfere with observed correlations (*12*) and the accuracy is also somewhat limited by the resolution in a 3D spectrum.

The 2D HMQC-J experiment (*13, 14*) relies on measurement of splittings in a resolution enhanced 2D $^1$H–$^{15}$N correlation spectrum. This experiment inspired us to implement *J*-multiplication (*15, 16*) for the measurement of homonuclear couplings using a variation of the sensitivity-enhanced $^1$H–$^{15}$N HSQC (*17*). The resulting spectrum, *J*-multiplied sensitivity enhanced HSQC (MJ-HSQC), allows direct observation of the $^3J(H_NH_\alpha)$ coupling constants with very good water suppression properties.

## DESCRIPTION OF THE PULSE SEQUENCE

The pulse sequence for MJ-HSQC is presented in Fig. 1.

The response of the MJ-HSQC sequence was analyzed with product operator calculations neglecting the signs, trigonometric factors, and homonuclear *J*-evolutions during the three INEPT steps (*20*). The sequence starts with a common homonuclear spin-echo sequence, where the delay $\tau$ is incremented in concert with $t_1$. The increment $\tau$ is a multiplet of $t_1$ increment ($N \times t_1$). This results in modulation of the signal by homonuclear coupling(s). If an $H^NH^\alpha N$ system with the initial state being $H_Z^NH_I^\alpha N_I$ (where I is the identity operator) is considered, the product operators after the $90_{(H)}$–$\tau$–$180_{(H)}$–$\tau$ propagator are of type $H_Y^NH_I^\alpha N_I$ and $H_X^NH_Z^\alpha N_I$. The first one of these gives rise to a signal that is cosine modulated with the desired *J*-coupling. The pulse sequence continues with an INEPT step ($\Delta = 1/(2^1J_{HN})$) and subsequent $t_1$ evolution. After the INEPT step, the operators are of the form $H_Z^NH_I^\alpha N_Y$ and $H_Y^NH_X^\alpha N_Y$. During the $t_1$-period only the $^{15}$N chemical shifts are active for these operators as the proton magnetization is refocused by the central $180_{(H)}$-pulse resulting in the operators $H_Z^NH_I^\alpha N_Y$, $H_Z^NH_I^\alpha N_X$, $H_Y^NH_X^\alpha N_Y$, and $H_Y^NH_X^\alpha N_X$. Both the *Y*- and *X*-components of the nitrogen magnetization are taken into account because of the sensitivity enhancement method. The $90_{(H)}90_{(N)}$ pulse pair converts these into $H_Y^NH_I^\alpha N_Z$, $H_Y^NH_I^\alpha N_X$, $H_Z^NH_X^\alpha N_Z$, and $H_Z^NH_X^\alpha N_X$. After the refocusing delay, $\Delta$, these have evolved into $H_X^NH_I^\alpha N_I$, $H_Y^NH_I^\alpha N_X$, $H_Z^NH_X^\alpha N_Z$, and $H_I^NH_X^\alpha N_Y$ ($H_Z^NH_X^\alpha N_Z$ is not affected as there is no large coupling between

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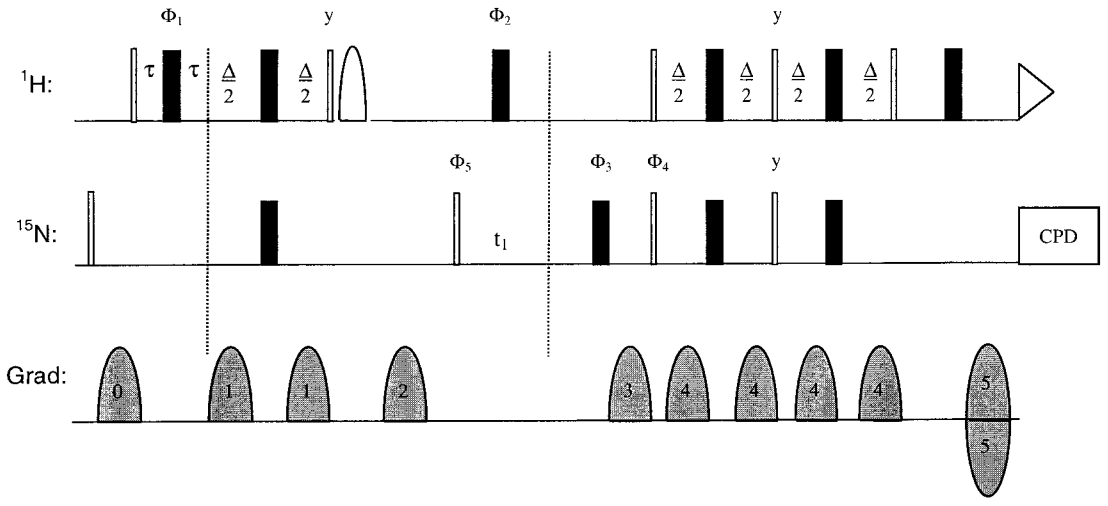
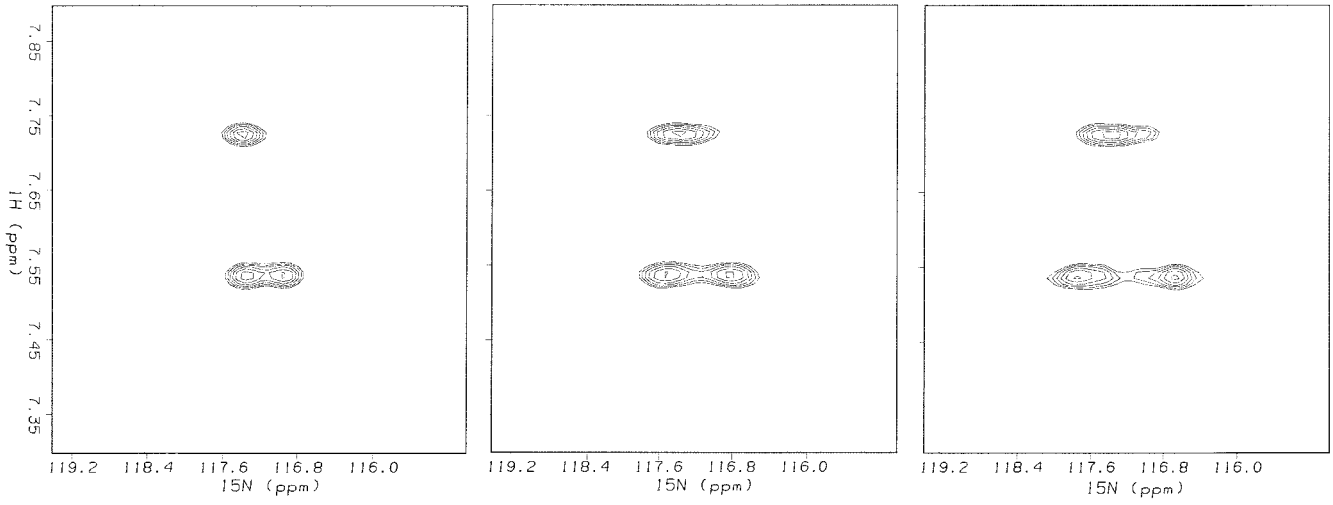



**FIG. 1.** Pulse sequence for the *J*-multiplied, sensitivity enhanced HSQC with gradient selection. Narrow white bars and wide black bars indicate 90 and 180 degree hard rectangular pulses. Gradient pulses are represented with grey half-ellipses and a selective 90 degree pulse on water resonance is represented by a white half-ellipse. All the pulses have *x*-phase unless otherwise indicated. Delay $\Delta = 1/(2\,{}^1J_{NH})$; the delay $\tau$ is incremented in steps of $N \times t_1$. Incrementation of $\tau$ in concert with $t_1$ results in the multiplication of ${}^3J(H_NH_\alpha)$ in the $f_1$-dimension. The phase cycle employed is $\Phi_1 = 8(x), 8(-x)$, $\Phi_2 = x, -x$, $\Phi_3 = x, x, y, y, -x, -x, -y, -y$, $\Phi_4 = x$, $\Phi_5 = x$, receiver $= x, x, -x, -x$. The N- and P-type coherences are recorded separately, which is achieved by simultaneously inverting the sign of gradient 5 and the phase of $\Phi_4$. Axial peak displacement is obtained via the States-TPPI method (*18*) by inverting the phase of $\Phi_5$ and receiver on every second increment. Decoupling of $^{15}N$ during the acquisition was performed using the WALTZ-16 sequence (*19*). The water suppression can be further improved using weak bipolar gradients (0.2 G/cm) during the $\tau$-periods to prevent the radiation damping (*24*).

$H_\alpha$ and $^{15}N$). The 90 degree pulses with *y*-phases on proton and nitrogen and the second $\Delta$ result in operators $H_Z^N H_I^\alpha N_I$, $H_X^N H_I^\alpha N_I$, $H_X^N H_Z^\alpha N_X$, and $H_Z^N H_Z^\alpha N_X$, which will be converted into $H_Y^N H_I^\alpha N_I$, $H_X^N H_I^\alpha N_I$, $H_X^N H_Y^\alpha N_X$, and $H_Y^N H_Y^\alpha N_X$ by the last $90_{(H)}$-pulse. Only the operators $H_Y^N H_I^\alpha N_I$ and $H_X^N H_I^\alpha N_I$ (cosine modulated by ${}^3J(H_NH_\alpha)$ in the $f_1$-dimension) contribute to observ-




| | | | |
|---|---|---|---|
| Multiplication factor (2N) | 3 | 5 | 7 |
| J(measured) [Hz] | 20.3 | 34.5 | 50.7 |
| J(actual) [Hz] | 6.8 | 6.9 | 7.2 |

**FIG. 2.** Expansion of the MJ-HSQC spectra of the Tec SH3 domain measured at 298 K using a 500 MHz Varian Unity spectrometer. Sample contained 0.5 mM protein and 20 mM sodium phosphate buffer in 97% $H_2O$/3% $D_2O$ at pH 6. Spectral widths were 8000 Hz ($^1H$) and 2100 Hz ($^{15}N$). The spectra were acquired with 1152 ($^1H$) $\times$ 200 ($^{15}N$) points and 16–128 scans per time increment. All the gradient pulses were block-shaped. Gradient 0: 4.0 G/cm, 1.000 ms, gradient 1: 5.0 G/cm, 0.750 ms, gradient 2: −15.0 G/cm, 1.500 ms, gradient 3: 30.0 G/cm, 1.250 ms, gradient 4: 4.0 G/cm, 0.500 ms, and gradient 5: 27.8 G/cm, 0.125 ms. A 90-degree shifted squared sine bell apodization function was used in both dimensions. After zero-filling the matrix size was 2048 $\times$ 1024 points. The correlation for residue Glu26 are shown ($\delta(^1H) = 7.54$ ppm). The upper correlation ($\delta(^1H) = 7.73$ ppm) originates from the side-chain amide proton of Gln16.

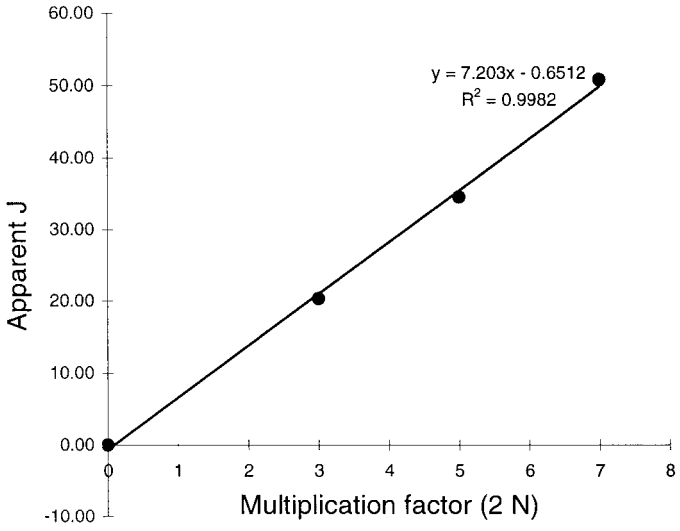



**FIG. 3.** Least-squares fitting of the apparent $^3J(H_NH_\alpha)$ coupling constants for residue Glu26, measured with different multiplication factors.

able proton signal and are processed like an echo–antiecho dataset to obtain pure absorptive in-phase signals. The observable signal will be split by $2N \times {}^3J(H_NH_\alpha)$ in the $f_1$-dimension ($N$ can be any positive number).

## RESULTS

We have tested the MJ-HSQC pulse sequence with the Tec SH3 domain (65 amino acids), which is currently under structure determination in our laboratory. In Fig. 2, a series of MJ-HSQC spectra acquired with increasing values of the multiplication factor are shown for residue Glu26 ($^1H$ $\delta = 7.54$ ppm). The increase in the apparent coupling constant is clearly visible. In Fig. 3 the least-squares fitting of measured coupling constants for this residue is presented. The line was fitted to three experimental data points (spectra run with multiplication factors $2N = 3$, 5, and 7) and extrapolated to $2N = 1$ yielding the true three-bond $J$-coupling. The extremely high $R$-factor shows, however, that a fitting procedure is usually not necessary, and that $^3J(H_NH_\alpha)$ values can simply be extracted from a single spectrum (measured, for example, with $2N = 3$). Obviously, the accuracy of the obtained coupling values will be increased (especially in case of small coupling constants), by the fitting procedure.

Table 1 lists some of the coupling constants of the SH3 domain of Tec, measured as mentioned above, together with values from a common COSY spectrum. In order to evaluate the accuracy of the MJ-HSQC experiment, we applied it to Ubiquitin, a protein with well-determined 3D structure. In Fig. 4, the MJ-HSQC spectrum of ubiquitin recorded using a multiplication factor of three ($2N = 3$) is presented. In Table 2 a set of $^3J(H_NH_\alpha)$ values measured with MJ-HSQC are compared with values obtained with HNHA (*12*) and with values

**TABLE 1**
**Comparison of Measured $^3J(H_NH_\alpha)$ Coupling Constants (Hz) for Some Residues of Tec SH3 Domain[a]**

| Residue | COSY | MJ method |
|---|---|---|
| M 12 | 7.33 | 5.90 |
| F 15 | 7.88 | 6.78 |
| A 17 | 6.09 | 4.46 |
| D 22 | 6.88 | 6.32 |
| L 23 | 8.30 | 8.15 |
| E 26 | 7.27 | 6.55 |
| R 27 | 9.38 | 8.34 |
| L 32 | 7.07 | 6.22 |
| I 33 | 9.36 | 9.02 |
| L 34 | 9.98 | 9.44 |
| V 39 | — | 3.00 |
| R 43 | 9.28 | 8.51 |
| A 44 | 9.81 | 8.91 |
| I 54 | 7.95 | 8.72 |
| N 57 | 6.52 | 5.73 |
| S 64 | 6.44 | 6.67 |

[a] $J$-values from the MJ-method were obtained as mentioned in the text, and values from the COSY spectrum were optimized by using the simulated annealing line fitting procedure implemented in the FELIX program (*21*). Out of these residues, D22, L23, L32–L34, R43, A44, and I54 are situated in typical $\beta$-sheet structures and the others in turning regions.

predicted from Karplus relationship (parametrized using the values of Vuister and Bax (*22*)) calculated from the X-ray structure (*23*).

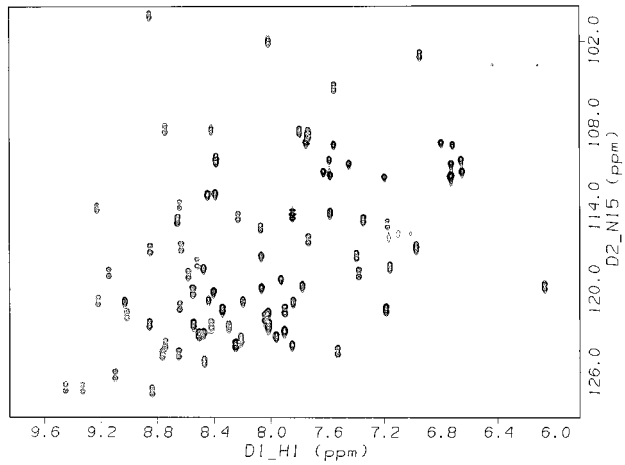



**FIG. 4.** The MJ-HSQC spectrum (multiplication factor = 3) of ubiquitin measured at 300 K, processed with a 90-degree shifted squared sine bell apodization function in the $f_2$-dimension (spectral width = 9600 Hz, number of points = 1024) and 70-degree shifted squared sine bell apodization function in the $f_1$-dimension (spectral width = 2000 Hz, number of time increments = 256). The spectrum was measured with two scans (relaxation delay = 1.0 s) and the total measurement time was about 17 min using a Bruker DRX 600 spectrometer. The $^{15}N$ labeled ubiquitin sample was obtained from VLI Research and the sample concentration was 1 mM (50 mM sodium phosphate buffer in 90% $H_2O$/10% $D_2O$ at pH 5.3).

**TABLE 2**
**Comparison of Measured $^3J(H_NH_\alpha)$ Coupling Constants of Some Residues of Ubiquitin[a]**

| Residue | MJ-HSQC | HNHA (*12*) | Karplus (*22, 23*) |
|---|---|---|---|
| E18 | 9.60 | 8.8 | 9.87 |
| K27 | 3.21 | 3.4 | 4.21 |
| D32 | 2.93 | 3.7 | 3.33 |
| R54 | 9.58 | 9.2 | 7.47 |
| I61 | 7.37 | 7.3 | 7.85 |
| H68 | 9.48 | 9.6 | 9.41 |

[a] Results from MJ-HSQC (averages from spectra acquired with multiplication factors $2N = 3$, 5, and 7), HNHA (*12*), and values calculated using the Karplus relationship from the X-ray structure (*23*).

## DISCUSSION AND CONCLUSIONS

Common methods for obtaining the $^3J(H_NH_\alpha)$ values in proteins are HNHA and HNCA-J. Whereas HNCA-J requires double-labeled protein samples, the HNHA method is applicable also for $^{15}$N-labeled samples. As both methods involve three-dimensional spectra, the time needed to perform these experiments can be quite long. The time needed to record a 2D MJ-HSQC spectrum is short and the suppression performance is very good. A drawback of the current method is line broadening in the $f_1$-dimension, which is caused by the simultaneous time incrementation during the $J$-coupling and shift evolution periods. In other words, the sampled $t_1$-increment for $^{15}$N shift corresponds to a period of $(2N + 1) \times t_1$ during which relaxation is active ($2N \times t_1$ for $T_2$ relaxation of amide protons). This problem becomes an issue with larger proteins as their $T_2$ relaxation times are short. Furthermore, with larger proteins, the achievable resolution in the $f_1$-domain will be low because the signal intensity drops relatively fast during the $t_1$-time incrementation. Thus, the limiting factor of the technique is the transverse relaxation of the amide protons. We estimate that current method will be applicable when amide proton linewidths are small ($\leq$10 Hz). This means it should work well for peptides and proteins up to 100 amino acid residues (the NH linewidths in the Tec SH3 domain were 7–10 Hz). One possibility to decrease the effect of the relaxation during the multiplication (delays $\tau$ in Fig. 1) is to create multiple quantum coherences. However, in our hands this did not give any improvement.

The accuracy of the current method (especially for small coupling constant values) can be improved by using resolution enhancement in $f_1$-domain and/or line fitting procedures for the in-phase doublets, as the apparent coupling tends to be smaller than the true value, when the two lines of the doublet overlap.

## ACKNOWLEDGMENT

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