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# An efficient 3D NMR technique for correlating the proton and $^{15}N$ backbone amide resonances with the α-carbon of the preceding residue in uniformly $^{15}N/^{13}C$ enriched proteins

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## SUMMARY

A 3D NMR technique is described which correlates the amide proton and nitrogen resonances of an amino acid residue with the Cα chemical shift of its preceding residue. The technique uses a relay mechanism, transferring magnetization from $^{15}N$ to $^{13}C\alpha$ via the intervening carbonyl nucleus. This method for obtaining sequential connectivity is less sensitive to large line widths than the alternative HNCA experiment. The technique is demonstrated for the protein calmodulin, complexed with a 26 amino acid fragment of skeletal muscle myosin light chain kinase.

Sequential resonance assignment of the backbone protons of a protein forms the basis for further solution structure studies by NMR. Commonly, this sequential assignment procedure relies heavily on through-space (NOE) connectivities. Alternatively, the potential for obtaining sequential assignment information through the use of J connectivities between backbone atoms has also been demonstrated (Llinas et al., 1977; Oh et al., 1988; Niemczura et al., 1989; Stockman et al., 1989). Recently, we developed a set of five 3D triple resonance NMR experiments (Kay et al., 1990) – relying on these J connectivities and not on NOEs – that made it possible to obtain complete $^{1}H$, $^{13}C$ and $^{15}N$ sequential backbone assignments for calmodulin (CaM) (Ikura et al., 1990), and for its complex with M13, a 26 amino acid fragment of myosin light chain kinase (Ikura et al.,

*Abbreviations:* CaM, Calmodulin; HCACO, α-proton to α-carbon to carbonyl correlation; H(CA)NHN, α-proton (via α-carbon) to nitrogen to amide proton correlation; HMQC, heteronuclear multiple quantum correlation; HNCA, amide proton to nitrogen to C α-carbon correlation; M13, a 26-residue fragment of the CaM-binding domain of skeletal muscle myosin light chain kinase comprising residues 577-602.

1991). This new procedure uses a combination of three 3D experiments, HCACO, H(CA)NHN and HNCA to correlate the backbone atoms within one amino acid residue. The HCACO experiment correlates Hα, Cα and the carbonyl resonance, C'. The H(CA)NHN experiment yields intraresidue connectivities between Hα, N and HN and relies on a magnetization transfer path from Hα to $^{15}$N which utilizes relay via the Cα resonance. In this experiment, transfer of magnetization from Cα to $^{15}$N via the one-bond $^{1}J_{NC\alpha}$ coupling ( ~ 11 Hz) competes with interresidue magnetization transfer via the two-bond $^{2}J_{NC\alpha}$ coupling which can be as large as −7 Hz. Both intra- and interresidue connectivities frequently can be observed in the H(CA)NHN spectrum provided the Cα line width is not much larger than the $J_{NC\alpha}$ couplings (Kay et al., 1991; Ikura et al., 1991).

The HNCA experiment correlates intraresidue HN, N and Cα resonances, but frequently also shows a connectivity between HN, N and the Cα resonance of the preceding residue, again by transfer via the two-bond $^{2}J_{NC\alpha}$ coupling. Indeed, in our recent study of the CaM-M13 complex 108 of the possible 145 intraresidue $^{1}$H-$^{15}$N-$^{13}$Cα connectivities were identified (Ikura et al., 1991). Thus, for 37 residues no interresidue connectivity was observed. Absence of these connectivities in some cases was due to overlap of the intraresidue and sequential HN-N-$C_\alpha$ correlations (14 residues) or rapid exchange with water of the amide proton (9 residues). For the remaining 14 missing connectivities, the intensity of this correlation was below the noise threshold, presumably caused by a small value of the $^{2}J_{NC\alpha}$ coupling. Observation of a two-bond H-N-$C_\alpha$ connectivity is particularly sensitive to the size of the $^{15}$N-$^{13}$Cα J coupling relative to the $^{15}$N line width, and as has been suggested, this type of correlation may become difficult to observe when the molecular correlation time, $\tau_c$, exceeds 10 ns (Kay et al., 1990).

Here we describe a different technique for obtaining the sequential HN-N-Cα connectivity, using a relayed transfer via the intervening carbonyl nucleus. This experiment circumvents the problem of accidental overlap of intra- and interresidue HN-N-Cα correlations, but more importantly, it is less sensitive to the $^{15}$N line width, i.e., the experiment is applicable to proteins with a correlation time significantly larger than 10 ns.

The pulse scheme of the new HN(CO)CA 3D experiment is sketched in Fig. 1. Using the pro-

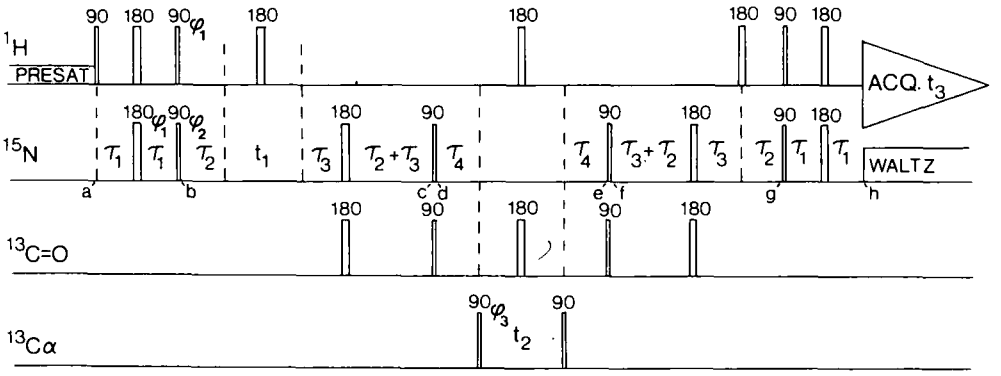


Fig. 1. Pulse scheme of the 3D HN(CO)CA experiment. All pulses for which the RF phase is not indicated are applied along the $x$ axis. The radiofrequency field strength of the carbonyl and Cα RF channels is adjusted such that a 90° Cα pulse causes minimal excitation in the carbonyl region and vice versa. Thus, at a $^{13}$C frequency of 150.9 MHz, the $^{13}$C 90° pulse width is set to 55 μs. The phase cycling scheme used is as follows: $\varphi_1$ = 2(y),2(−y); $\varphi_2$ = x,−x; $\varphi_3$ = 4(x),4(−x); Rec. = x,2(−x),x,−x,2(x),−x. Quadrature in the $t_1$ and $t_2$ dimensions is obtained using the States-TPPI method (Marion et al., 1989a): for each ($t_1$; $t_2$) pair $\varphi_2$ and $\varphi_3$ are independently incremented by 90° and data are stored in separate locations.

duct operator formalism (Ernst et al., 1988) the relevant magnetization transfer steps are outlined below. For clarity, relaxation terms are not included and any terms that do not result in observable magnetization during the acquisition time, $t_3$, are omitted in this description as are constant multiplicative factors preceding these terms. The following spin operator nomenclature is used: I for the HN proton spin, N for the $^{15}N$ spin, and S for the carbonyl spin. Longitudinal HN magnetization, described by $I_z$ and present at time *a* is described by a term $\sigma_a = I_z$. This magnetization is transferred into antiphase $^{15}N$ magnetization using an INEPT transfer (Morris and Freeman, 1979) giving at time *b*:

$$\sigma_b = N_y I_z \sin(2\pi J_{NH}\tau_1) \tag{1}$$

At time *c*, the antiphase term present in $\sigma_b$ has been refocused and simultaneously dephasing has occurred due to J coupling between $^{15}N$ and the carbonyl resonance, S. If the delay $\tau_2$ is adjusted to exactly $1/(4J_{NH})$, the relevant magnetization term at time *c* is described by:

$$\sigma_c = N_y S_z \sin(2\pi J_{NH}\tau_1)\sin(\pi J_{NS}(2\tau_2 + 2\tau_3 + t_1))\cos(\Omega_N t_1) \tag{2}$$

where $\Omega_N$ is the angular $^{15}N$ frequency and $J_{NS}$ is the $^{15}N$-carbonyl J coupling. In deriving this expression, J coupling between $^{15}N$ and $^{13}C\alpha$, active during the evolution period $t_1$, is neglected. At time *c*, the pair of simultaneous 90° $^{15}N$ and carbonyl 90° pulses transfers $\sigma_c$ in an INEPT-like manner into antiphase carbonyl magnetization. Temporarily omitting the cosine terms of expression (2), one finds:

$$\sigma_d = S_y N_z \tag{3}$$

This magnetization remains antiphase with respect to the $^{15}N$ spin but is correlated with the C$\alpha$ spin, A, in an HMQC-style manner (Bendall et al., 1983; Bax et al., 1983). Neglecting J coupling between C$\alpha$ and $^{15}N$ during the time $t_2$, and between the carbonyl and protons during the time $t_2 + 2\tau_4$, one obtains at time *e*:

$$\sigma_e = S_y N_z \sin^2(\pi J_{SA}\tau_4)\cos(\Omega_A t_2) \tag{4}$$

where $J_{SA}$ denotes the one-bond C'-C$\alpha$ J coupling. The following INEPT-type transfer converts the antiphase carbonyl magnetization into antiphase $^{15}N$ magnetization. Again omitting the sine and cosine terms of expression (4) one finds at time *f*:

$$\sigma_f = N_y S_z \tag{5}$$

During the subsequent interval $2\tau_2 + 2\tau_3$, $^{15}N$ magnetization becomes in-phase with respect to the carbonyl spin, S and antiphase with respect to the amide proton, I. Thus, at time *g* one obtains:

$$\sigma_g = N_y I_z \sin(\pi J_{NS}(2\tau_2 + 2\tau_3)) \tag{6}$$

Finally, the last INEPT sequence transfers this magnetization into observable in-phase transverse amide magnetization of the form:

$$\sigma_h = I_x \sin^2(2\pi J_{NH}\tau_1)\sin(\pi J_{NS}(2\tau_2 + 2\tau_3 + t_1))\cos(\Omega_N t_1) \times \sin(\pi J_{NS}(2\tau_2 + 2\tau_3))\sin^2(\pi J_{SA}\tau_4)\cos(\Omega_A t_2) \quad (7)$$

where all the relevant sine and cosine terms have been reintroduced. Values of the delays $\tau_1$, $\tau_2$, $\tau_3$ and $\tau_4$ have to be optimized in a manner described by Kay et al. (1991). Assuming $J_{NH} \approx 92$ Hz, $J_{NC'} \approx 15$ Hz, $J_{C'C\alpha} \approx 55$ Hz, $^1J_{NC\alpha} \approx 11$ Hz and $^2J_{NC\alpha} \approx 7$ Hz, near optimum values for the $\tau$ delays are: $\tau_1 = 2.25$ ms; $\tau_2 = 2.75$ ms; $\tau_3 = 8$ ms; $\tau_4 = 7$ ms. In order to avoid resolving the $J_{NC'}$ coupling in the $F_1$ dimension (which would lead to a loss in signal-to-noise ratio obtainable per unit of measuring time) the acquisition time in the $t_1$ dimension, $t_{1_{max}}$, is kept relatively short, such that $t_{1_{max}} + 2\tau_2 + 2\tau_3$ is smaller than $1/J_{NC'}$. Similarly, in order to avoid sensitivity loss caused by the $^1J_{C\alpha C\beta}$ splitting in the $F_1$ dimension, the acquisition time in the $t_2$ dimension, $t_{2_{max}}$, must be kept shorter than $1/(2J_{C\alpha C\beta})$.

The pulse scheme of Fig. 1 utilizes refocused INEPT schemes (Burum and Ernst, 1983) to transfer magnetization from the amide $^1H$ to $^{15}N$ and back. Use of refocused INEPT requires one more 180° pulse but no extra delays compared to an optimized pulse scheme (not shown) that utilizes antiphase INEPT transfers. Because refocused INEPT schemes are used, $\sigma_d$ of expression (3) does not depend on $I_z$ and therefore relaxation of the magnetization present between time points *d* and *e* in Fig. 1 does not depend on the relaxation rate of $I_z$. Note that the relaxation rate of $I_z$ may be quite short because in a macromolecule this rate is determined by spin diffusion (London, 1990) and this rate may also be enhanced by exchange of the amide proton with solvent. The improved relaxation behavior probably more than offsets the loss of magnetization introduced by the additional (imperfect) 180° pulse required for the refocused INEPT.

The experiment is demonstrated for a sample containing uniformly $^{15}N$ and $^{13}C$ enriched calmodulin (CaM), complexed with unlabeled M13 peptide. The molecular mass of the complex is 19.7 kDa. The concentration of the sample was 1.5 mM in 95% $H_2O$, 5% $D_2O$, 100 mM KCl, 25°C, pH 6.95. Preparation of this sample has been described elsewhere (Ikura et al., 1991). Spectra were recorded on a Bruker AM600 spectrometer equipped with additional synthesizers, gating circuitry, phase shifters and power amplifiers for generating the RF pulses for the two 'non-Bruker' channels (C′ and Cα). A similar hardware set-up has been described in detail elsewhere (Kay et al., 1990).

Measurement of the transverse relaxation time using a 1–1 echo scheme (Sklenar and Bax, 1987) which suppresses homonuclear J modulation showed an average $T_2$ value of 24.8 ms at 35°C for the group of overlapping amide protons at ~9 ppm. A preliminary $^{15}N$ relaxation study indicates a correlation time of ~10 ns for the CaM-M13 complex at this temperature. When the temperature was reduced to 25°C, the amide $T_2$ decreased to 19.8 ms. Assuming that transverse relaxation is due to a combination of dipolar couplings and $^1H$ chemical shift anisotropy and that the transverse relaxation rate of the overlapping HN protons is directly proportional to the spectral density at zero frequency, J(0), this suggests a correlation time, $\tau_c$, of about 12-13 ns at 25°C.

Figure 2 shows two ($F_2$,$F_3$) slices taken from some of the most crowded regions of the 3D HN(CA)CO spectrum, showing interresidue correlations between the HN of residue *i* and the Cα of residue *i* − 1. The spectrum was recorded in about 40 h and the high signal-to-noise ratio indi-

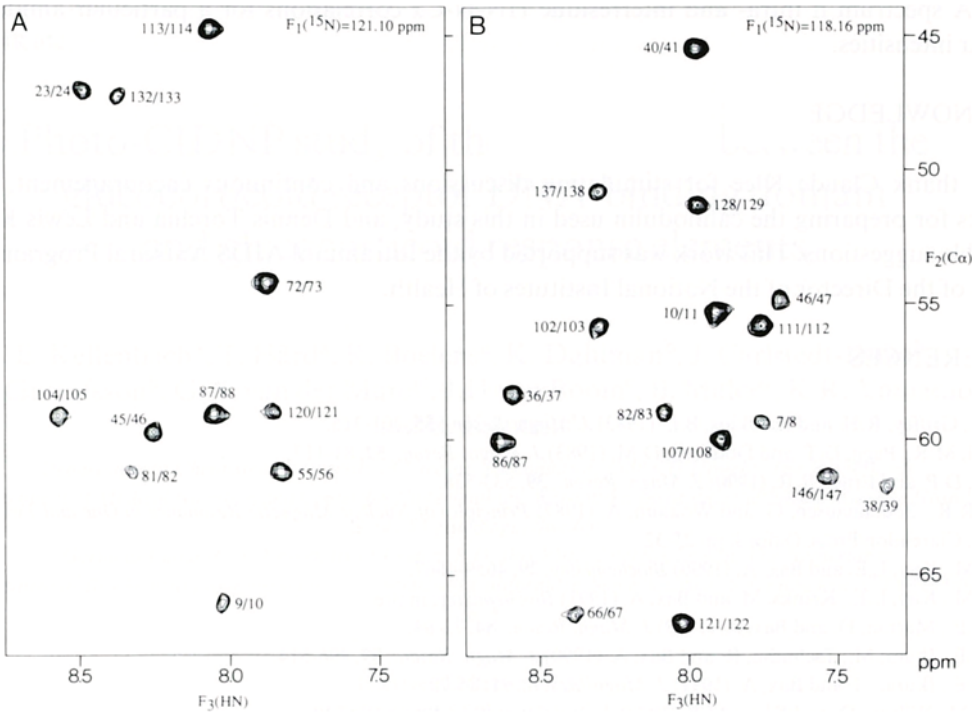


Fig. 2. Two ($F_2$,$F_3$) slices taken from the HN(CO)CA spectrum of the CaM-M13 complex, recorded at 600 MHz. The slices display interresidue connectivities between amide protons of residue *i* and Cα resonances of $i-1$. The spectrum results from a (32 complex) × (64 complex) × (1024 real) data matrix, with acquisition times of 22.4 ms ($t_1$), 12.8 ms ($t_2$) and 51.2 ms ($t_3$). After shifted sine bell apodization, zero filling and Fourier transformation, the absorptive part of the 3D matrix consists of 64 × 56 × 1024 data points. For each set of ($t_1$,$t_2$) values, 64 transients were recorded (including the States-TPPI phase cycling) and presaturation of the $H_2O$ resonance with a 25 Hz RF field was used during the delay of 0.95 s between scans. The total measuring time was ~40 h. Data were processed with the package NMR2 (NMRi, Syracuse, NY) supplemented by home-written software for decreasing baseline distortions introduced by residual $H_2O$ (Marion et al., 1989b) and for Fourier transformation and digital filtering in the $F_1$ dimension (Kay et al., 1989).

cates that a protein concentration significantly lower than the 1.5 mM used in our study would have been sufficient for providing spectra of adequate quality. While the slices shown in Fig. 2 may suggest that there is substantial variation in the intensities of the HN-N-Cα correlations, in fact, this is not the case. The intensity variation visible in these slices is caused mainly by the fact that not all of the $^{15}N$ frequencies correspond exactly to the $F_1$ frequency at which the slice is taken, and correlations that are weak in Fig. 2 are more intense in adjacent slices.

Out of the possible 145 interresidue HN-N-Cα correlations, 139 were actually observed in the 3D spectrum. No correlations were observed to the amides of D2, Q3, N42, D78, T79 and S81 because for these residues the amide hydrogen exchange rate is too fast ($k_{HX} > 30\ s^{-1}$) at the pH and temperature used in the present study (Spera et al., in press).

The technique described in this communication provides a sensitive and powerful tool for obtaining sequential connectivity information which is complementary to that obtainable with other 3D triple resonance experiments. The method is less sensitive to large linewidths than the alternative HNCA experiment and also solves ambiguities that sometimes arise during analysis of the

HNCA spectrum if intra- and interresidue HN-N-Cα correlations for a particular amide show similar intensities.

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