

COMMUNICATIONS

Rapid Recording of 2D NMR Spectra without Phase Cycling. Application to the Study of Hydrogen Exchange in Proteins

DOMINIQUE MARION,* MITSUHIKO IKURA, ROLF TSCHUDIN, AND AD BAX

*Laboratory of Chemical Physics, National Institute of Diabetes and Digestive and Kidney Diseases,
National Institutes of Health, Bethesda, Maryland 20892*

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Although the inherent sensitivity of 1D and 2D NMR spectroscopy is not very different (1), the minimum time needed for recording a 2D NMR spectrum is typically many orders of magnitude longer. This stems from the requirement to sample a sufficiently large number of t_1 increments, necessary to obtain adequate digital resolution in the F_1 dimension of the final spectrum. Moreover, the pulse sequence is usually repeated many times for each t_1 value, with different phases of the RF pulses, in a process referred to as phase cycling. This phase cycling is used to eliminate spurious resonances such as axial peaks and quadrature artifacts from the 2D spectrum, or to select desired coherence transfer pathways.

Here we demonstrate that high quality 2D NMR spectra can be recorded very rapidly, without using phase cycling. This can result in dramatic time savings for commonly used sensitive ^1H experiments such as COSY, HOHAHA (2, 3), or HMQC (4-6) and makes it possible to study, on a relatively rapid time scale, ^1H exchange phenomena in proteins with spectra too complex for analysis by 1D NMR.

In the absence of phase cycling, it is important to minimize the inherent sources of spectral artifacts. Quadrature artifacts in the F_2 dimension can largely be eliminated by proper balancing of the two receiver channels. F_1 quadrature artifacts can originate from imperfect RF phase shifts. These are minimal on modern NMR spectrometers equipped with digital phase shifters. Other F_1 artifacts can result from too short a delay time between scans; i.e., the longitudinal magnetization at the beginning of the pulse sequence depends on the outcome (and thus on the RF phases and t_1 duration) of the previous scan. Depending on the order of the steps used in a phase cycle, these repetition rate artifacts can be attenuated strongly by phase cycling. In the absence of phase cycling, it is important to have the same initial (usually incompletely relaxed) state at the beginning of each pulse sequence. To this extent, we apply a saturation pulse (7) at the end of every t_2 data acquisition period. In practice, we find a nonselective $10000_x^\circ - 5000_y^\circ$ pulse pair adequate for effectively scrambling all magnetization. This procedure has the undesired side effect of annihilating the magnetiza-

* On leave from the Centre de Biophysique Moléculaire CNRS, 1A Av. de la Recherche Scientifique, 45071 Orléans Cedex 2, France.

tion recovery that occurs during the data acquisition period. For sequences such as HOHAHA that employ a trim pulse at the end of the mixing period, the spin system always starts from a state with no z magnetization at the beginning of t_2 . In this case, no saturation pulse is needed at the end of the t_2 period.

If no phase cycling is to be used, the pulse sequence needs to be analyzed critically with respect to possible undesired coherence transfer pathways that could result from imperfect 180° pulses. As a general rule, to minimize artifacts originating from this source, pulse widths should be calibrated carefully and all 180° pulses applied during the evolution period of 2D experiments should be of the composite type (8).

The main remaining problem is the suppression of axial peaks from the 2D spectrum. These peaks result from magnetization that is not modulated during the time t_1 , i.e., from magnetization that is longitudinal at the end of the t_1 period. The magnitude of this longitudinal magnetization is independent of the preparation period pulse phases. Therefore, if all t_1 modulation frequencies are artificially increased by using time proportional phase incrementation (TPPI) of these pulses (9), the axial peak signals remain unmodulated. After the real Fourier transformation, normally used in TPPI type spectra, the axial peaks appear at the $F_1 = 0$ edge of the 2D spectrum. Unless these axial peaks are extremely intense, they do not interfere with the interpretation of the resonances of interest. If they do interfere, their presence is easily eliminated by treatment of the data with a time-domain convolution difference procedure in the t_1 dimension (10).

The TPPI method has the disadvantage relative to the method described by States *et al.* (11) that folding properties are less desirable, that phasing may be problematic (12), and that shifting of the F_1 carrier frequency after the data have been acquired is not possible (13). For this reason, we prefer use of the States method of acquisition in our 3D NMR experiments (13). Note, however, that with this method, axial peaks (in the absence of phase cycling) will appear in the center of the spectrum, where they commonly overlap with resonances of interest. Instead, we propose to use a combination of the States and TPPI methods: For each t_1 duration the results of the x and y experiments are stored in separate locations, and each time t_1 is incremented, the phases of all preparation pulses and of the receiver are inverted. Because of the change in receiver phase by 180° for every t_1 increment, it appears as if the axial signals invert their sign every t_1 increment, i.e., as if they are modulated by the Nyquist frequency. All other signals remain unchanged relative to the regular States method. Consequently, axial peaks are moved to the edge of the spectrum.

In this respect, it is important to note that the position of the axial peaks is not dependent on whether the x and y components of complex t_1 data points are sampled simultaneously or sequentially (separated by one dwell time) as originally proposed by Redfield and Kunz (14). The difference only originates in the time proportional phase incrementation which moves the position of the spectrum with respect to the axial peaks. The different ways of obtaining quadrature information in the F_1 dimension and the corresponding position of axial peaks are indicated in Table 1.

Using the approach described above to circumvent phase cycling, 2D spectra can be recorded in a very short time. The rapid acquisition technique is demonstrated for two experiments: a HOHAHA experiment using 15 mg BPTI, freshly dissolved in

TABLE I
Axial Peak Position for Different Ways of F_1 Quadrature Detection

Method	Prep. ϕ	Receiver	Fourier transform	Axial peak
Redfield	$x(t_1)$	x	Real	Center
	$y(t_1 + \Delta)^a$	x		
	$x(t_1 + 2\Delta)$	-x		
	$y(t_1 + 3\Delta)$	-x		
TPPI	$x(t_1)$	x	Real	Edge
	$y(t_1 + \Delta)$	x		
	$-x(t_1 + 2\Delta)$	x		
	$-y(t_1 + 3\Delta)$	x		
States	$x(t_1)$	x	Complex	Center
	$y(t_1)$	x		
	$x(t_1 + 2\Delta)$	x		
	$y(t_1 + 2\Delta)$	x		
TPPI-States	$x(t_1)$	x	Complex	Edge
	$y(t_1)$	x		
	$-x(t_1 + 2\Delta)$	-x		
	$-y(t_1 + 2\Delta)$	-x		

^a The time, Δ , equals $1/(2SW_1)$, where SW_1 is the F_1 spectral width.

0.5 ml D₂O, p²H 3.7, 24°C, and a ¹H-detected ¹H-¹⁵N shift correlation experiment of uniformly ¹⁵N-labeled calmodulin, freshly dissolved in D₂O, 1.5 mM, p²H 5.8, 100 mM KCl, 6 mM Ca²⁺, 35°C. The spectra are recorded on a Bruker AM-500 spectrometer, modified to permit buffered acquisition; i.e., no overhead time is needed between scans for bookkeeping or writing data to the disk.

Figure 1a shows the HOHAHA spectrum of BPTI, recorded in 5 min, using the TPPI-States method and results from a $2 \times 128 \times 1024$ data matrix, with two scans to obtain each complex t_1 data point. The delay time between scans was 1.1 s, and the acquisition times were 20.7 ms (t_1) and 83 ms (t_2). Acquisition of the 2D matrix was started 8 min after the sample was dissolved in D₂O. As has been shown before for COSY and NOESY spectra, the fingerprint (NH-C α H) regions of such maps are extremely useful to follow the amide hydrogen exchange process (15, 16). An expansion of the fingerprint region of the HOHAHA spectrum is shown in Fig. 1b. In the present case, protons that exchange with the deuterated solvent at a rate $>0.2 \text{ min}^{-1}$ are attenuated more than 10-fold in the 2D spectrum. Correlations for residues G12, K15, A25, R42, N43, D50, R53, and G57 with exchange rates in the range 0.05–0.2 min^{-1} are clearly observable.

Many proteins are not soluble to such a high concentration (4.5 mM) as was used above. Consequently, the sensitivity of HOHAHA or COSY spectra may be too low for obtaining the required signal-to-noise ratio in such a short period of time. Moreover, for larger proteins the increased linewidth further reduces the sensitivity of the ¹H-¹H J correlation methods and even the 2D ¹H-¹H correlation spectrum may become too overlapped for detailed analysis. For cloned and overexpressed proteins,

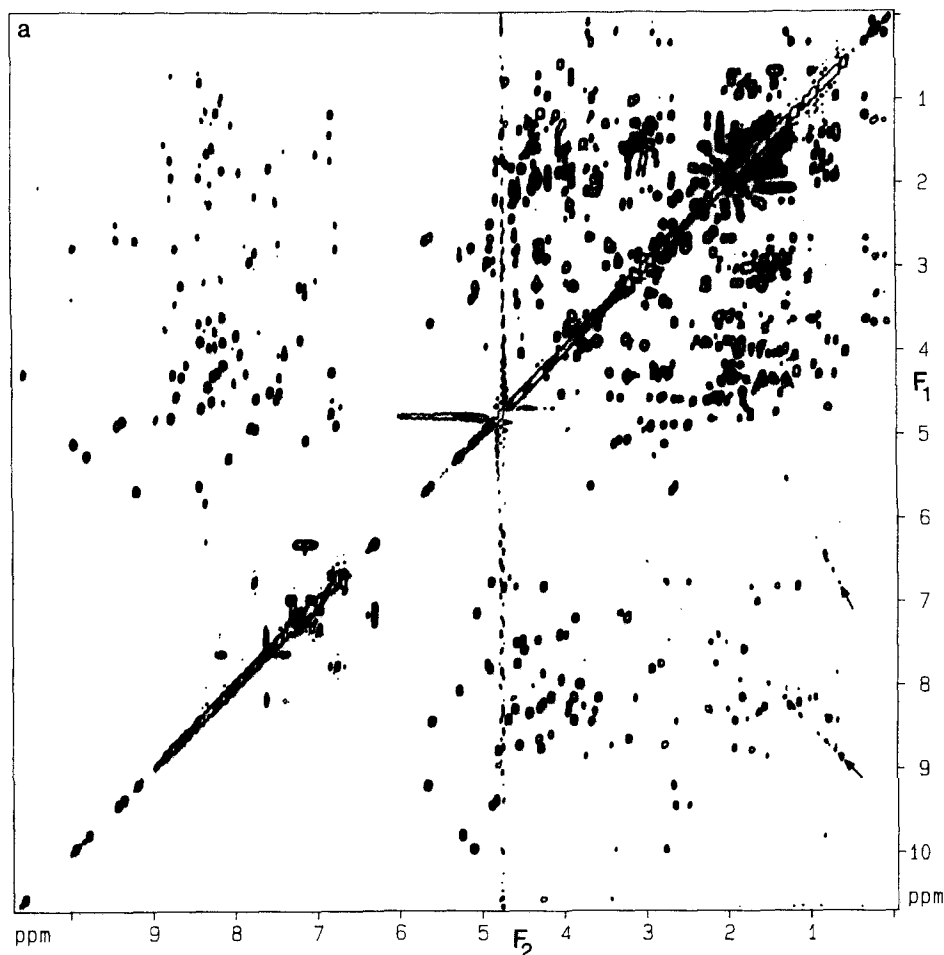


FIG. 1. The 500 MHz 2D HOHAHA spectrum of 15 mg BPTI, freshly dissolved in 0.5 ml D_2O , p^2H 3.7, $24^\circ C$. The spectrum has been recorded using a WALTZ mixing scheme with a total duration of 35 ms, preceded and followed by 1 ms trim pulses. The spectrum has been recorded with the TPPI-States method of data acquisition and results from a $2 \times 128 \times 1024$ data matrix, with acquisition times of 20.7 ms (t_1) and 83 ms (t_2). The total measuring time was 5 min. A 60° -shifted sine square bell filter (t_2) and a 45° -shifted sine bell filter (t_1) were used. Zero filling was employed to yield a digital of 6 Hz in both dimensions. The residual HDO resonance was attenuated by presaturation. (a) The resonance containing portion of the 2D spectrum. The entire 2D spectrum extends from 10.9 to -1.4 ppm, with the carrier positioned on the HDO resonance. Weak antidiagonal artifacts, resulting from too short a delay time between scans, are marked by arrows. (b) An expansion of the fingerprint region, with assignments taken from Otting and Wüthrich (19). Previously observed but unidentified resonances are marked "?".

a more sensitive alternative is available: it is relatively straightforward and inexpensive to label such proteins with ^{15}N , and to record 1H - ^{15}N shift correlation spectra. The pulse sequence first proposed by Bodenhausen and Ruben (17), and often referred to as the Overbodenhausen experiment (6, 18), provides a good compromise of sensitivity and resolution. The sequence is

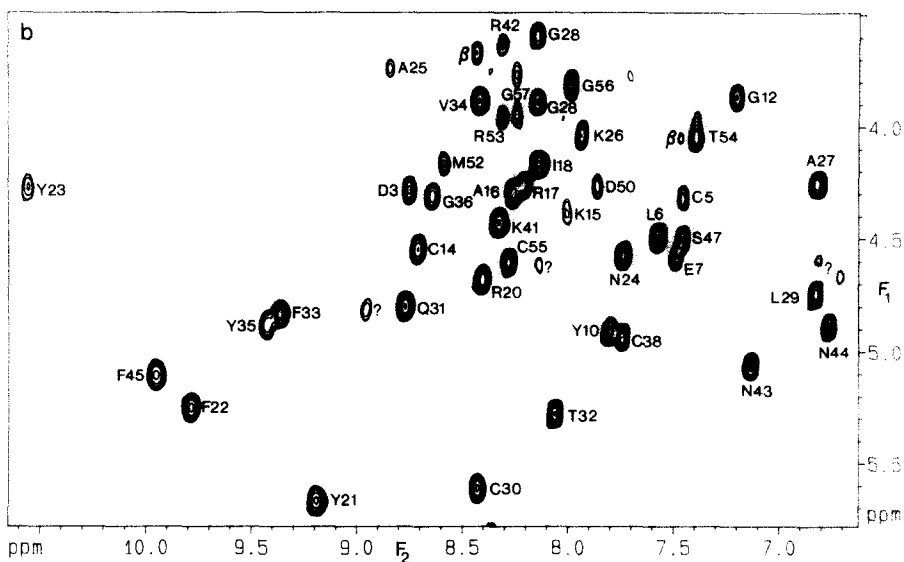


FIG. 1—Continued

$$^1\text{H}: 90_x^\circ - \Delta - 180_x^\circ - \Delta - 90_y^\circ - t_1/2 - 90_x^\circ 240_y^\circ 90_x^\circ - t_1/2 - 90_x^\circ -$$

$$^{15}\text{N}: 180_x^\circ \quad 90_\phi \quad 90_x^\circ$$

$$\Delta - 180_x^\circ - \Delta - \text{Acq.} - 10000_x^\circ 5000_y^\circ$$

$$180_x^\circ \quad \text{Decouple}$$

The delay Δ is set to $1/(4J_{\text{NH}})$. The phase of the receiver is kept constant and ϕ is incremented by 90° in the TPPI or TPPI-States fashion. The pulse at the center of the evolution period is of the composite type to minimize artifacts in the final spectrum. Note that signal from protons not coupled to ^{15}N is not suppressed in this experiment because of the absence of phase cycling. Most of this signal will not be modulated as a function of t_1 because ^1H chemical-shift effects are refocused by the composite 180° pulse at the center of t_1 . The very small fraction of ^1H signals that are not refocused give rise to a weak diagonal at $F_1 = F_2$. Because these signals do not experience the TPPI incrementation of ϕ , this diagonal is offset in the F_1 dimension by $SW_1/2$, where SW_1 is the F_1 spectral width. Magnetization from weak RF regions in the sample that is longitudinal during the first half of t_1 is incompletely inverted by the composite 180° pulse and gives rise to a "half-diagonal" at $F_1 = 0.5F_2$. By judicious choice of the carrier positions in the F_1 and F_2 dimensions, overlap with the spectral region of interest can be avoided.

Figure 2 shows the region of the ^1H - ^{15}N shift correlation map containing all unexchanged amide proton correlations. The spectrum was recorded with the Overbodenhausen scheme (TPPI) and results from a 256×1024 data matrix, corresponding to acquisition times of 77 and 83 ms in the t_1 and t_2 dimensions, respectively. The ^1H

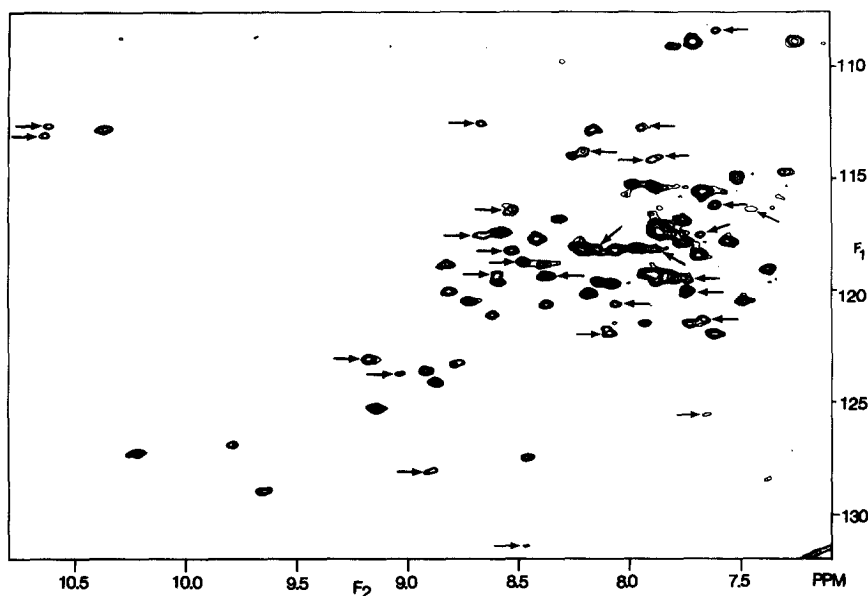


FIG. 2. ^1H - ^{15}N correlation spectrum of a 1.5 mM solution of uniformly ^{15}N -labeled calmodulin (16.7 kDa) recorded with the Overbodenhausen pulse scheme. The total measuring time was 5 min and the experiment was started 4 min after the protein was dissolved. Correlations absent in a similar spectrum recorded 12 min later are marked by arrows. The spectrum has been recorded using the TPPI method of F_1 quadrature detection. Mild resolution enhancement (Lorentz-Gauss) and zero filling to 6 Hz digital resolution (F_2) and 45° -shifted sine bell filtering and zero filling to 3 Hz digital resolution (F_1) were used. No baseline correction or other cosmetic procedures were used.

carrier was positioned at 8.3 ppm and the ^{15}N carrier at 121 ppm, and SW_1 (33 ppm) was chosen to cover the entire amide ^{15}N spectral width (132–105 ppm). The measuring time was 5 min, and the experiment was started 4 min after the sample was dissolved in D_2O . Approximately half of the NH protons are absent from this spectrum, indicating that they already have exchanged completely (>85%) with solvent deuterons. Resonances that disappear from the shift correlation map in a spectrum recorded 10 min later (data not shown) are marked in the figure. Complete amide assignments of this protein and a quantitative analysis of the hydrogen exchange rates will be presented elsewhere.

We have shown that 2D NMR spectra may be recorded exceptionally rapidly and that no phase cycling is needed provided that experiments are properly optimized. Of course, a requirement for the rapid recording of spectra is that the inherent sensitivity of the particular experiment is high and sample concentrations are such that a 2D spectrum may be obtained with only a single transient per t_1 value (TPPI) or two transients per t_1 value (TPPI-States). This is often the case for COSY, HOHAHA, or reverse correlation spectra of small organic molecules and as shown here, even for biological macromolecules it may be possible to record 2D spectra very rapidly.

The reduction in experimental measuring time obtainable by the omission of phase cycling is particularly important for 3D NMR experiments that require high resolu-

tion in both F_1 and F_2 dimensions. With phase cycling, these experiments can necessitate measuring times of more than one week. The absence of a two-step axial peak suppression phase cycle in each of these dimensions reduces the minimum measuring time fourfold.

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