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Three-Dimensional Homonuclear Hartmann–Hahn–Nuclear Overhauser Enhancement Spectroscopy in H_2O and Its Application to Proteins

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The potential of three-dimensional NMR spectroscopy to extend the methodology of protein structure determination by NMR has recently been demonstrated (*1*). Peaks, generally referred to as relayed peaks in 2D NMR, are spread out into 3D frequency space, thereby reducing the extent of spectral overlap. Because the location of a 3D cross peak in 3D space is described by the chemical shifts of three different spins, i, j, k, the resolution of a 3D spectrum is superior to that of the corresponding 2D relayed spectrum. The key to protein structure determination by NMR lies in the identification of through-bond and through-space connectivities (*2, 3*). Particularly important are those involving the exchangeable NH protons. Hence it is necessary to work in H_2O. In our previous paper (*1*) we presented a 3D nuclear Overhauser enhancement (NOESY)–homonuclear Hartmann–Hahn (HOHAHA) spectrum of the protein α_1-purothionin in 90% H_2O. In this particular sequence, suppression of the water resonance other than by irradiation is technically very difficult, as well as prohibitive in measurement time due to the extensive phase cycling required (*4*). As a result, many correlations involving $C^{\alpha}H$ protons that resonate in the vicinity of the water resonance are eliminated and signals of rapidly exchanging NH protons are abolished by transfer of saturation. To overcome these problems, we have designed a novel 3D HOHAHA–NOESY pulse sequence in which the water resonance is suppressed using a jump–return read pulse (*5*) and frequency selection in the second dimension is achieved using a semiselective z filter (*6*). The superiority of this sequence over the previous one (*1*) is illustrated using α_1-purothionin as an example.

General schemes for 3D NMR spectroscopy consist of combining two 2D NMR techniques [e.g., COSY (*7*), NOESY (*8*), HOHAHA (*9, 10*)] leaving out the detection period of the first experiment and the preparation period of the second (*1, 11, 12*). To reduce the size of the 3D data matrix and yet retain sufficiently well-resolved spectra, the two evolution times t_1 and t_2 may be preceded by semiselective soft pulses to reduce the spectral widths in the first ($F1$) and second ($F2$) dimensions (*11–13*).

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The pulse scheme we propose for the 3D HOHAHA–NOESY experiment is shown in Fig. 1. By placing the NOESY unit at the end of the 3D pulse sequence, suppression of the water resonance using a jump–return (*5*) read pulse becomes relatively straightforward. This advantage, however, must be traded for difficulties in frequency selection in $F2$ as transverse magnetization of the whole spectrum remains after the mixing process of the HOHAHA unit. In order to reduce the spectral width in $F2$, the coherences of the signals occurring in the spectral regions outside the desired spectral range must be converted into z magnetization, otherwise fold-over of these signals occurs. It can be demonstrated experimentally that the coherences that evolve with frequencies lying outside the desired envelope must be destroyed almost perfectly, as they correspond to peaks known as diagonal peaks in 2D NMR spectroscopy. Consequently, even small residuals of the coherences are very intense. We have solved this problem by applying a pulse unit which we term a "semiselective z filter" (*6*). After the HOHAHA part of the sequence, a nonselective 90° pulse is applied to create z magnetization, followed by a short delay during which a moderate field gradient is used to destroy residual transverse magnetization. A semiselective pulse then excites the desired coherences prior to the evolution period t_2. The longitudinal magnetization, created by the first pulse of the semiselective z filter, may be selected by more or less elaborate phase cycling of all the subsequent pulses to achieve the desired removal of all artifacts, as is usually the practice with z filters. Antiphase terms created during the spin-locking period, for example, would be canceled by such phase cycling.

We found, however, that the application of a field gradient was also important. This is because the creation of z magnetization by the "flip-back" pulse (i.e., the first pulse of the semiselective z filter) is imperfect and the number of phase cycles possible is limited by the need to record the 3D NMR spectrum in a reasonable time. As a result of the inclusion of the semiselective z filter, all the peaks in the spectrum have positive in-phase lineshapes with no negative elongations. This approach has the additional advantage that the frequency changes needed to select the desired spectral range of the full 3D spectrum always occur in periods during which the finally selected magnetization is situated along the z axis. This avoids delays in which free precession

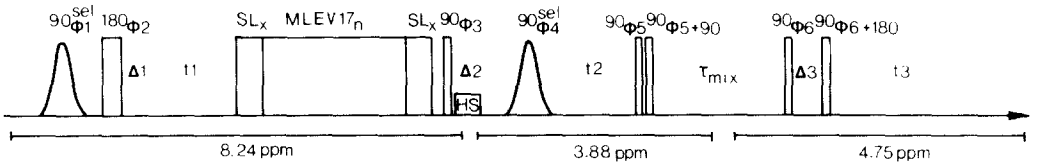

FIG. 1. Pulse sequence for 3D HOHAHA–NOESY in H_2O. The first unit—a semiselective HOHAHA experiment—is followed by the semiselective z filter which prepares the desired coherences for the NOESY unit. To refocus the chemical-shift precession during the first soft pulse (Gaussian-shaped), a π pulse is applied before t_1 together with a suitable delay. This approach could not be used after the second soft pulse, because the necessary additional phase cycling would require too many scans per increment. The length of the delay Δ_1 should be approximately one-half of the length of the Gaussian pulse. At 500 MHz, the length of the delay Δ_3 is typically 80 μs. The position of the transmitter in our experiment is given in parts per million below the sequence. The phases are cycled as follows: $\varphi 1 = 2(x), 2(-x)$; $\varphi 2 = 2(x, -x)$; $\varphi 3 = 2(y, y, -y, -y), 2(-y, -y, y, y)$; $\varphi 4 = 4(x), 4(-x)$; $5 = x$; $\varphi 6 = 16(x), 16(-x)$; receiver $= 4(x), 8(-x), 4(x), 4(-x), 8(x), 4(-x)$.

of transverse magnetization occurs and, as an added bonus, permits the recording of 3D NMR spectra on NMR spectrometers that cannot change frequency in a coherent manner.

In the α_1-purothionin example presented here, the region comprising the NH protons is excited with the first soft pulse and the region of the $C^{\alpha}H/C^{\beta}H$ protons with the second. Thus, cross peaks with three different coordinates result from the pathway

$$\mathrm{NH} \xrightarrow[+\text{relayed } J]{J} \mathrm{C^{\alpha}H/C^{\beta}H} \xrightarrow{\mathrm{NOE}} \mathrm{NH/aliphatics.}$$

The 3D NMR spectrum obtained for α_1-purothionin using this sequence is similar to that obtained with the NOESY-HOHAHA sequence (*1*) in the subvolume comprising the NH($F1$)-$C^{\alpha}H/C^{\beta}H$($F2$)-NH($F3$) region ($F1$ = 6.3–10.2 ppm, $F2$ = 1.9–5.8 ppm, $F3$ = 6.2–9.6 ppm; Fig. 2), but very different in the subvolume com-

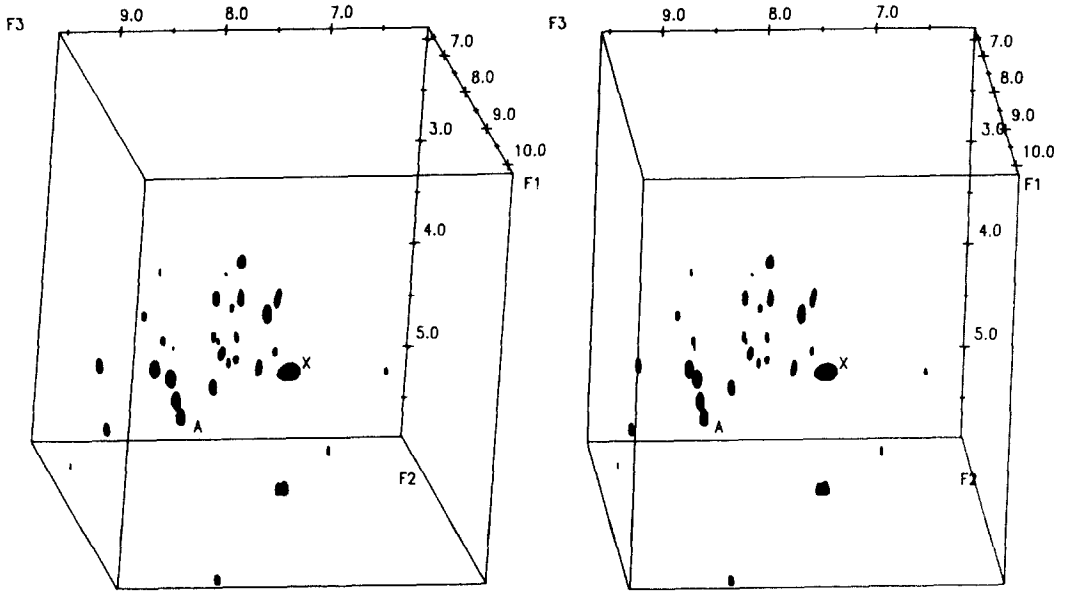

FIG. 2. Stereoview of the NH($F1$)-$C^{\alpha}H/C^{\beta}H$($F2$)-NH($F3$) subvolume of the 3D HOHAHA-NOESY spectrum of α_1-purothionin (6.7 mM) in 90% H_2O/10% D_2O. The spectrum was recorded in 55 h on a Bruker AM 500 spectrometer, equipped with a selective excitation unit and a Tokyo-HL30 linear amplifier; 64 × 64 × 2048 data points were collected for a spectral width of 2000 × 2000 × 7042 Hz in $F1$, $F2$, and $F3$, respectively, and 32 transients were acquired per increment. The length of the Gaussian pulses was 3 ms at a truncation level of 1%. The mixing time of the HOHAHA part was 44 ms and that of the NOESY, 220 ms. The processed portion of the spectrum shown consists of 128 × 128 × 256 real points. Appropriate Lorentz-to-Gauss transformations were applied together with polynomial baseline corrections of degrees <6 in all three dimensions. The spectrum was processed on a Convex C-1 computer in ~10 min—including the baseline corrections—and displayed on an Evans & Sutherland PS 390 interactive graphics system using the program FRODO (*16*). At the contour level displayed, 12 cross peaks of the type NH(i) → $C^{\alpha}H(i)$ → NH(i + 1) are visible; the others are intraresidue cross peaks. The peak marked with an X is the only artifact in the spectrum and arises from an unavoidable spurious excitation of the huge water signal with the first soft pulse. This corresponds to a strong cross peak in the 2D NOESY between all the Lys-NH_3^+ protons and the water resonance. Peak A is discussed in the text.

prising the NH($F1$)-C$^{\alpha}$H/C$^{\beta}$H($F2$)-aliphatic($F3$) region ($F1$ = 6.3–10.2 ppm, $F2$ = 1.9–5.8 ppm, $F3$ = 0.4–3.8 ppm; Fig. 3). In the case of the NOESY–HOHAHA sequence, the latter subvolume mainly contains cross peaks that arise from through-bond connectivities involving the C$^{\alpha}$H and C$^{\beta}$H protons of individual amino acids. In the present spectrum, however, cross peaks are due to NOEs between C$^{\alpha}$H protons and aliphatic side-chain protons providing the C$^{\alpha}$H and NH protons belonging to the same residue exhibit a $^3J_{HN\alpha}$ coupling of reasonable size (>5 Hz).

Figure 2 shows the region 6.3–10.2($F1$)/1.9–5.8($F2$)/6.2–9.6($F3$) ppm of the HOHAHA–NOESY spectrum. The capital "A" indicates a cross peak with coordinates 8.89($F1$)/4.71($F2$)/8.76($F3$) ppm corresponding to the connectivity $Ser_2NH \rightarrow Ser_2C^{\alpha}H \rightarrow Cys_3NH$). This corrects the previously assigned NH resonance of Ser_2 (*14*), which was mistakenly identified at 7.82 ppm from a 2D HOHAHA spectrum due to the presence of t_1 noise arising from the intense water resonance. Fortunately, this correction has no effect on the results of the 3D structure determination of α_1-purothionin as no NOEs involving the Ser_2NH proton were used in the calculations (*15*). Proof for the identity of the Ser_2NH resonance at 8.89 ppm can be derived from cross peaks B and C in the subvolume shown in Fig. 3. Cross peak B gives the connection NH–C$^{\alpha}$H–C$^{\beta 2}$H for Ser_2 with the frequency coordinates 8.89($F1$)/4.71($F2$)/2.84($F3$) ppm; cross peak C arises from the pathway $Ser_2NH \rightarrow Ser_2C^{\alpha}H \rightarrow Ile_{33}C^{\delta}H_3$. The latter cross peak represents a further tertiary NOE contact between Ser_2 and Ile_{33}, in addition to that between the β proton of Ser_2

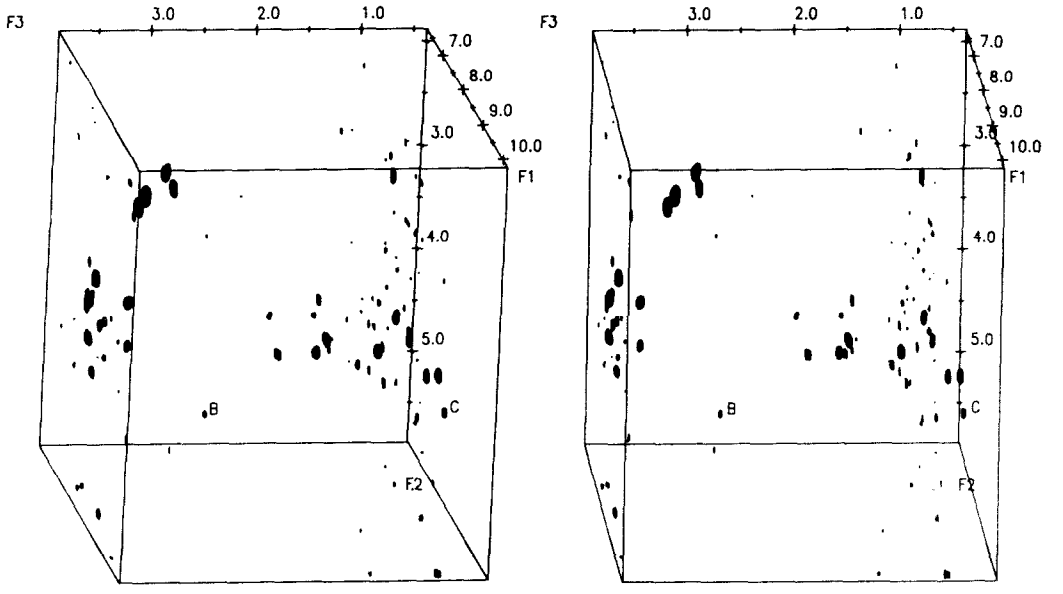

FIG. 3. Stereoview of the NH($F1$)-C$^{\alpha}$H/C$^{\beta}$H($F2$)-aliphatic($F3$) subvolume of the 3D HOHAHA–NOESY spectrum of α_1-purothionin in 90% H_2O. At the contour level displayed, 28 peaks can be identified. The majority arise from intraresidue NOEs. Some, however, arise from interresidue NOEs of considerable value, as discussed in the text for peak C.

and the δ-methyl group of Ile_{33} previously identified in the 2D NOESY spectra (*14, 15*).

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