New Carbon-Detected Protein NMR Experiments Using CryoProbes

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The sensitivity of NMR experiments depends critically on the type of nucleus that is detected during acquisition. It increases with $\gamma^{3/2}$, where γ is the gyromagnetic ratio of the detected nucleus. In theory this dependence increases the sensitivity of proton-detected experiments over carbon-detected experiments by a factor of 8 and over nitrogen-detected experiments by more than a factor of 30. Consequently, carbon-detected experiments have been restricted to small organic molecules containing few protons and to solid state applications. In contrast, virtually all modern biomolecular NMR pulse experiments detect protons during acquisition.

The very recent introduction of cryogenic probes[1,2] into the field of high-resolution NMR has increased the sensitivity of NMR experiments by a factor of 3−4. While the relative sensitivity of proton detection and carbon detection is the same as for a conventional probe head, carbon-detected NMR experiments can now be brought into a sensitivity range that makes them feasible even for biomolecular applications. For the past decade biomolecular NMR pulse sequence development has almost exclusively focused on proton detection. The availability of these cryoprobes and hence the possibility of carbon detection opens a new area for technique development that broadens the tools available for the investigation of structure and dynamics of biological macromolecules.

Carbon detection offers certain unique advantages over proton detection. One is the possibility of directly detecting carbons that lack covalently bound protons, for example, carbonyls. Another advantage is that the need for water suppression and the unavoidable artifacts it causes are eliminated in ^{13}C-detected NMR experiments. This is particularly important for experiments that detect α-protons, which have chemical shifts similar to that of water.

The theoretical loss of a factor of 8 in carbon-detected experiments can be at least partially compensated by additional advantages of carbon detection. First of all, ^{13}C-detected pulse sequences are shorter than ^{1}H-detected pulse sequences, due to the elimination of one or more INEPT transfer steps. ^{13}C-detected experiments also use a smaller number of radio frequency pulses, minimizing losses due to off-resonance effects and B_1-field inhomogeneity. Moreover, carbons without directly attached protons often show favorable relaxation characteristics[3−5] that make them attractive for direct detection. As an example of how to use these specific advantages of carbon detection to design experiments with high sensitivity we will discuss a version of the HCACO[6−8] experiment with direct detection of the carbons.

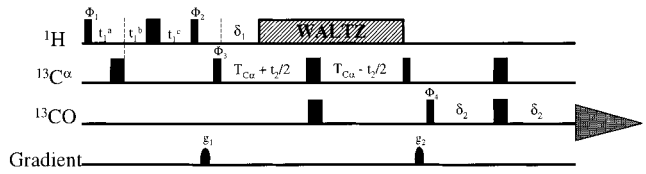

Figure 1. Pulse scheme for the carbonyl-detected HCACO experiment. Narrow and wide bars represent 90° and 180° pulses, respectively. The ^{13}C pulses were applied with a field strength of 3.6 kHz for the 90° pulses and 7.2 kHz for the 180° pulses. The values of t_1^a, t_1^b, t_1^c, δ_1, $T_{C\alpha}$ and δ_2 were set to 1.5 ms, 3 μs, 1.5 ms, 3.5, 4.5, and 4.5 ms. Proton decoupling was achieved with a waltz16 sequence (3.1 kHz field strength). The duration and strengths of the gradient pulses were: $g1 = (345\ \mu s, 5$ G/cm) and $g2 = (300\ \mu s, 8$ G/cm). Phase cycling: $\Phi_1 = (x,-x)$, $\Phi_2 = (2y, 2-y)$, $\Phi_3 = (4x, 4-x)$, $\Phi_4 = (8x, 8-x)$, receiver = $(x, 2-x, x, -x, 2x, 2-x, 2x, -x, x, 2-x, x)$.

Figure 1 shows the pulse sequence of the carbonyl-detected HCACO experiment. It starts with an INEPT transfer from the α-proton to α-carbon. To optimize the sensitivity of the experiment, during this INEPT step the α-proton is frequency-labeled in a semiconstant-time manner.[9] In the subsequent delay $2T_{C\alpha}$ the ^{13}Cα−^{1}Hα coupling is refocused and the coupling between the ^{13}Cα and the ^{13}CO spin evolves. This delay is also used for frequency labeling of the ^{13}Cα spins in a constant-time manner. At the end of the delay $2T_{C\alpha}$ coherence is transformed into carbonyl coherence that is antiphase with respect to the directly bound ^{13}Cα spin. This coupling is refocused during the delay $2\delta_2$, followed by detection of carbonyl magnetization during acquisition.

The original proton-detected version of the HCACO is an "out-and-back" type of experiment.[6−8] It starts with equilibrium α-proton magnetization and detects α-proton magnetization during acquisition. This requires two INEPT steps to transfer coherence from the α-proton to the α-carbon and back to the α-proton. In addition, two INEPT steps for the evolution and refocusing of the ^{13}CO−^{13}Cα coupling are necessary. During all these INEPT steps the relevant product operators contain transverse Hα or Cα operators. Because the α-proton and the α-carbon have short relaxation times, minimizing the delays during which the Hα and Cα coherences evolve can be used to at least partially compensate the loss of a factor of 8 in sensitivity. This is achieved in the pulse sequence shown in Figure 1. The carbonyls are detected, and the original "out-and-back" HCACO experiment becomes an "out-and-stay" version, eliminating the INEPT step that transfers coherence from the α-carbon back to the α-proton. This reduces the time during which fast-relaxing Hα coherences exist by a factor of 2. The sensitivity is further increased by refocusing the ^{13}CO−^{13}Cα antiphase coherence into a carbonyl in-phase coherence rather than an α-carbon coherence. This replaces a delay involving fast-relaxing Cα coherences with a delay involving

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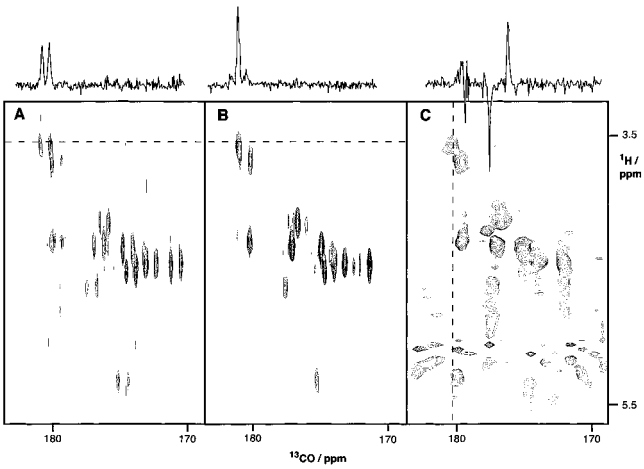

Figure 2. Section of a ^{1}Hα−^{13}CO plane from 3D HCACO experiments with a 0.6 mM sample of a ^{13}C-labeled 17 kD fragment of the *E. coli* protein Ada. Only positive contour levels are shown. The experiment was measured on a Bruker Avance 500 MHz spectrometer equipped with a 5 mm ^{13}C−^{1}H dual CryoProbe with *z*-gradients. Each experiment was measured with a total of 21 increments in the ^{13}Cα dimension and 25 increments in the second indirect dimension with 32 scans per increment. The measurement time was 24 h. (A) Section taken from a carbonyl-detected HCACO. The carbonyl acquisition dimension was processed using Fourier transformation, showing the ∼55 Hz coupling between the ^{13}Cα and the ^{13}CO spins. (B) The acquisition dimension was transformed and the coupling deconvolved using maximum entropy reconstruction. (C) Section taken from a conventional proton-detected HCACO[7] experiment that used coherence selection (without sensitivity enhancement) at the end of the ^{13}Cα evolution delay for water suppression. The 1D slices on top of each section are taken along the acquisition dimension at the position indicated by the dashed lines. The spectral width of the 1 D slice shown in C is larger than the spectral width of the 2D plane in C to allow a better representation of the noise level.

slower-relaxing carbonyl coherences. An additional advantage of the pulse sequence shown in Figure 1 is the smaller number of pulses compared to the original proton-detected sequence. The biggest contribution to sensitivity, however, is the replacement of the fast-relaxing α-protons with the slower-relaxing carbonyl spins during acquisition. The slower relaxation of the carbonyls[3−5] makes it worthwhile to increase the acquisition time, which results in higher sensitivity.

Water suppression is, of course, unnecessary in carbonyl-detected experiments. This is particularly important for the HCACO experiment because the α-proton chemical shifts overlap with the water resonance. The elimination of any water suppression scheme in the pulse sequence in Figure 1 greatly reduces artifacts.

Figure 2A shows a typical ^{1}Hα−^{13}CO plane from a 3D carbonyl-detected HCACO experiment that was measured with a 0.6 mM sample of a 17 kD fragment of the *Escherichia coli* protein Ada in 24 h. Each peak is split into a doublet due to the ^{13}CO−^{13}Cα coupling of ∼55 Hz that evolves during the acquisition. The evolution of this coupling can, in principle, be suppressed by selective decoupling of the ^{13}Cα spins. Alternatively, the coupling can be removed from the spectra after the acquisition by deconvolution, which avoids artifacts and possible loss in sensitivity associated with time-shared decoupling. This is possible since the ^{13}CO−^{13}Cα coupling is very uniform. Figure 2B shows the same plane as 2A, but instead of Fourier transformation along the acquisition dimension, t_3 has been processed using maximum entropy reconstruction.[10] This method is one of several that can be used to deconvolve the coupling without the need for homonuclear decoupling during acquisition. On top of each spectrum a 1D slice along the acquisition dimension is shown, taken at the position indicated by the dashed line. Comparison of slices A and B demonstrates that deconvolution leads to an increase in the signal/noise ratio of almost the full theoretical factor of 2.

Figure 2C shows a ^{1}Hα−^{13}CO plane from a conventional HCACO[7] experiment that was measured with proton detection on a TXI-CryoProbe that corresponds to the plane from the carbonyl-detected experiment shown in Figure 2A and 2B. Despite the use of coherence selection for water suppression, a large number of artifacts are visible around the water resonance position, partially overlapping with the cross-peaks and making interpretation difficult. In contrast, the carbonyl-detected spectrum is virtually free of artifacts. Comparison of the 1D slices B and C further shows that the sensitivity of the carbonyl-detected and the proton-detected experiment are comparable. This demonstrates that the advantages of carbon detection discussed above can largely compensate for the original loss of a factor of 8 in sensitivity.

The principle of converting an "out-and-back" pulse sequence into an "out-and-stay" type of experiment with the help of carbon detection during acquisition is not limited to the HCACO experiment. The same strategy that is outlined above can, for example, be applied to the HCACON[6−8] experiment (with detection on the carbonyls) and the HCACO[N]−E.COSY[11] experiment. Furthermore, detection of carbonyls during acquisition will be useful for carbonyl relaxation experiments and for the determination of residual dipolar couplings involving the carbonyls. The backbone carbonyls are not the only spins that can be used for detection during acquisition. Detection of side chain carbonyls and aromatic carbons can be useful for the selective identification of aromatic amino acids and residues with side-chain carbonyls. Carbon detection also offers new strategies for pulse sequence developments for structure determination of nucleic acids, which have a small number of protons. In addition, experiments with highly deuterated proteins could benefit from carbon detection.

Many triple resonance experiments start with amide proton magnetization and detect it during acquisition. Although experiments such as the HNCACO[12] or HNCO[6,13] can easily be transformed into an "out-and-stay" version with carbonyl detection, the slower relaxation of the amide protons compared to the α-protons makes it more difficult to compensate the sensitivity loss due to carbon detection. However, at high pH values the fast chemical exchange between amide protons and water can severely reduce the sensitivity of experiments involving amide protons. Carbon-detected experiments are less susceptible to signal loss caused by fast chemical exchange with water because they do not require water suppression and because carbons do not exchange. A careful design of the pulse sequence can, therefore, almost eliminate signal loss caused by saturation transfer from the water protons. If the pulse sequence does not start with amide proton magnetization but with aliphatic protons, the sensitivity becomes nearly unaffected by chemical exchange and, therefore, almost independent of pH. For investigations of proteins at high pH, a combination of amide-proton-detected and carbon-detected experiments might, therefore, be useful.

In conclusion, direct carbon detection, made practical by cryogenic probes, can be a complementary or alternative technique to established proton-detected experiments when detection of carbons without directly attached protons is advantageous, water suppression is problematic, or chemical exchange between amide protons and water is fast. In these cases a careful design of the pulse sequence can often at least partially compensate the loss of a factor of 8 in sensitivity due to carbon detection.

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