Carbon-Detected NMR Experiments To Investigate Structure and Dynamics of Biological Macromolecules

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We have started to develop new NMR pulse sequences that detect carbon magnetization during the acquisition period. These experiments have become possible with the recent introduction of cryogenic probe heads. We show that a careful design of these carbon-detected experiments can at least partially compensate for the inherent lower sensitivity of carbon detection compared to proton detection. We discuss potential applications of carbon detection and demonstrate a deconvolution technique that removes the effects of carbon – carbon couplings from the spectra.

KEYWORDS:

NMR spectroscopy · protein structures · pulse sequences · structure elucidation

Introduction

During the last two decades the demand for stronger magnets to increase the sensitivity of NMR experiments has dominated the development of new NMR spectrometers. The very recent introduction of cryogenic probe heads in the field of high-resolution NMR spectroscopy, however, has opened a new route to achieve high sensitivity even at relatively low/weak magnetic fields.[1–3] In these cryogenic probe heads the receiver coil and part of the electronics is cooled to a few kelvins which reduces the noise and, therefore, enhances the sensitivity of the probe head. Depending on the sample an increase by a factor of 3 – 4 in the signal-to-noise ratio can be readily achieved. Practically, this means that a certain NMR experiment will become more sensitive by this factor of 3 – 4. Alternatively, to reach the same sensitivity as with a conventional probe head, the measurement time can be reduced by a factor of 9 – 16.[1]

Cryogenic probe heads not only increase the sensitivity of conventional NMR experiments, but also allow the development of new NMR techniques. A large class of NMR experiments, consisting of pulse sequences that detect carbon rather than proton magnetization, has long been considered too insensitive for biomolecular applications. Virtually all modern biomolecular NMR experiments detect proton magnetization during acquisition due to the dependence of the sensitivity on the gyromagnetic ratio of the detected nucleus, γ. The sensitivity increases with $\gamma^{3/2}$, making carbon- and nitrogen-detected experiments less sensitive by a factor of 8 and 30, respectively, relative to proton-detected experiments.[4–7] Consequently, carbon detection has been so far restricted to small organic molecules with few protons and to solid-state applications. Although cryogenic probe heads do not change the relative sensitivity of the different nuclei, they push the absolute sensitivity of carbon-detected experiments into a range that makes them attractive even for biomolecular applications.[3, 5] In this article we want to discuss the advantages and disadvantages of carbon detection and present some applications of this technique.

Detection on carbon atoms that lack covalently bound protons

Proton detection is, of course, only possible if protons are present in a molecule. While biological macromolecules usually provide a high density of protons, they also contain carbon atoms that are not covalently attached to protons. To obtain information about these carbon atoms with conventional proton-detected experiments, the magnetization has to be relayed through at least one additional heteronucleus (carbon or nitrogen) that has directly attached protons. In contrast, carbon detection allows for direct observation of the carbon magnetization during acquisition and, therefore, simplifies the pulse sequences. In proteins, carbon atoms without covalently attached protons include main-chain and side-chain carbonyl groups, the ζ-carbon atom of arginines, the γ-carbon atoms of all aromatic amino acids as well as the ξ-carbon atom of tyrosines and the $\varepsilon 2$- and $\delta 2$-carbon atoms of tryptophan. Nucleic acid bases also contain a number of carbon atoms without directly attached protons, including carbonyl groups in cytosine, thymine, uracil, and guanine, the C4 atom in cytosine, C5 in thymine, C4, C5, C6 in adenine, and C2, C4, and C5 in guanine. Direct detection of these carbon atoms offers an

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attractive new method for obtaining chemical shift information or for investigating macromolecular dynamics.

Deuteration of carbon atoms is an important technique that allows investigation of large (>30 kDa) proteins by NMR spectroscopy[8–11] and high levels of deuteration can lead to additional carbon atoms without directly attached protons. In perdeuterated proteins the only protons present are the amide protons, forcing all proton-detected experiments to transfer the magnetization to them for detection during acquisition.[8] For these highly deuterated proteins, carbon detection offers additional possibilities for pulse sequence development.

Solvent suppression

While the concentration of water in an aqueous NMR sample is 55 M (or 110 M in terms of protons), the concentration of the biological macromolecule of interest is usually in the 0.5 – 5 mM range. This enormous difference in concentration leads to a water proton signal that is several orders of magnitude larger than the signal of the macromolecule. The signal that is received by the coils in the probe head has to be converted into a digital signal that can be stored in a computer with the help of an analog-to-digital converter (ADC).[4] Digitization of a very small signal in the presence of a large one, however, reduces the sensitivity for the small signals. For this reason, many different water suppression techniques that range from presaturation with a weak radio frequency field to gradient selection methods have been designed that aim at reducing the water signal to levels comparable to the signals of the dissolved macromolecules.[4, 12–14] Although some of the currently available methods almost completely eliminate the water signal,[14] they all produce spectral artifacts that are usually manifested as a noise band at the position of the water signal along the indirect detection dimension. These noise bands are a particular problem in NMR experiments that detect α protons. Their chemical shift range overlaps with the water signal and their signals can be obscured by artifacts produced by water suppression. In contrast to proton detection, carbon-detected experiments do not require any form of water suppression, which also eliminates all associated artifacts. Carbon detection becomes even more advantageous for samples that are dissolved in aqueous protonated buffers, such as acetate, Tris or HEPES buffer. Although their concentration range is 20 – 50 mM—considerably smaller than the concentration of water—the long relaxation times of the small organic molecules make a complete suppression impossible, causing additional artifacts in the spectrum. While the natural abundance of the ^{1}H proton isotope is almost 100%, the natural abundance of the NMR-active carbon isotope ^{13}C is only 1.1%, which reduces the signals and artifacts produced by the buffer molecules considerably in carbon-detected experiments.

Sensitivity

As mentioned in the Introduction, carbon detection is less sensitive than proton detection by a theoretical factor of eight. This huge loss in sensitivity has so far prevented most applications of carbon detection. However, a careful design of the pulse sequence combined with certain advantages of carbon detection can at least partially compensate for this sensitivity loss. In general, carbon-detected experiments are shorter than proton-detected ones due to the elimination of one or more magnetization transfer steps that are necessary to bring the magnetization back to protons prior to detection.[3] This shortening of the pulse sequence reduces the signal loss caused by relaxation, which is particularly important for the investigation of large macromolecules. In addition, the elimination of transfer steps also reduces the number of radio frequency pulses in the NMR experiment, which minimizes losses due to off-resonance effects and B_1-field inhomogeneity. As an example, we show a direct comparison of a conventional proton-detected HCCH-TOCSY experiment and a carbon-detected version. This experiment is used to assign the side-chain resonances of proteins by transferring the magnetization throughout the side chain.[15, 16] The first part of the two pulse sequences that are shown in Figure 1 is identical and consists of a series of steps that transfers

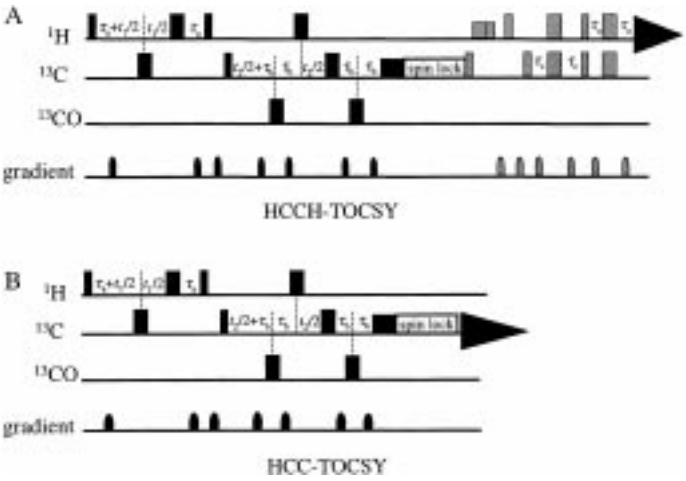

Figure 1. *Comparison of the pulse sequences of a conventional proton-detected HCCH-TOCSY (A) and a carbon-detected HCC-TOCSY experiment (B). 90° radio frequency pulses are shown as narrow bars, 180° pulses as wide bars, and gradient pulses as shapes on the line-labeled gradient. Pulses and gradients in Figure A that are omitted from the pulse sequence in Figure B are shown in gray.*

the magnetization form protons to carbon atoms. During the spin lock period, this carbon magnetization is transferred to other carbon spins within the same side chain, which creates characteristic peak patterns in the resulting NMR spectra that can be used to assign the side-chain resonances. In the conventional HCCH experiment, this spin lock period is followed by a water suppression module that consists of two orthogonal proton trim pulses and two strong gradient pulses.[16] The magnetization is then transferred through two steps back to protons for detection. In contrast, in the carbon-detected experiment, the acquisition period starts right after the end of the spin lock period. This eliminates the entire water suppression module as well as the two magnetization transfer steps including a total of eight radio frequency pulses. Similar reductions in time

and number of pulses can be achieved with other pulse sequences.

The sensitivity of carbon-detected NMR experiments can be further increased if carbon spins without directly attached proton spins are detected during acquisition. These carbon spins show favorable relaxation characteristics due to their reduced dipole – dipole interaction with protons.[17–19] Their slow relaxation makes it worthwhile to increase the acquisition time, which results in a higher sensitivity.

Furthermore, the absence of a large signal like the water signal in carbon-detected experiments allows one to utilize the maximum sensitivity of the analog-to-digital converter for the digitization of the signal, which further increases the sensitivity of these experiments.

Carbon – carbon coupling and deconvolution

Figure 2 shows the first planes of a conventional, proton-detected HCCH-TOCSY (Figure 2 A) and of a carbon-detected HCC-TOCSY experiment (Figure 2 B). These spectra demonstrate

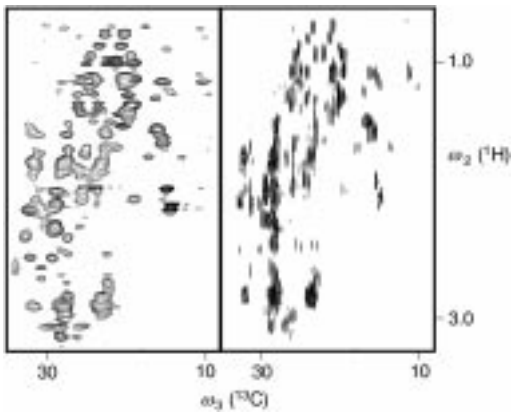

Figure 2. *First plane taken from a proton-detected HCCH-TOCSY (A) and from a carbon-detected HCC-TOCSY experiment (B) with a 0.8 mM sample of a 14-kDa fragment of the transcription factor Cdc5.[25] In the HCC-TOCSY experiment, each peak is split by the carbon – carbon coupling in at least two individual lines. Each experiment was measured with eight scans per increment, 1024 complex points in the acquisition dimension, and 64 complex points in the indirect dimension. All experiments described in this work were measured on a Bruker Avance 500 NMR spectrometer equipped with either a 5-mm $^{13}C-^{1}H$ dual Cryoprobe or a triple-resonance Cryoprobe.*

that carbon-detected experiments can yield acceptable sensitivities. However, inspection of the HCC-TOCSY spectrum reveals that all peaks are split into two or more individual lines. This splitting results from the carbon – carbon coupling that evolves during the acquisition period. This coupling is quite uniform between different types of aliphatic carbon atoms and leads to a splitting of ca. 35 Hz between the individual lines of a multiplet.[20] The effect can be seen best in the methyl region (around $\delta = 10$ in the carbon dimension). Methyl group carbon atoms have only one neighboring carbon atom and their signals are, therefore, split into a doublet. The pattern for carbon atoms with couplings to two or three other carbon atoms is more complicated. The distribution of the signal intensity into two or more individual lines reduces the sensitivity of the experiment. Fortunately, data processing techniques have been developed that can remove the splitting from the spectra, collapse the individual lines of a multiplet, and restore the full sensitivity of the experiment.[21] In the following we will discuss the HCACO experiment as a specific example of how to use data processing techniques to remove the splitting. In this context we will also show how the advantages of carbon detection can be combined to create highly sensitive NMR experiments.

The HCACO experiment

The HCACO experiment is a standard NMR experiment that is used to assign the backbone signals of proteins.[22–24] It correlates the frequencies of the α-proton with the frequencies of the α-carbon atom and of the carbonyl group. The pulse sequence of the conventional proton-detected experiment is shown in Figure 3 A and the schematic representation of the carbon-

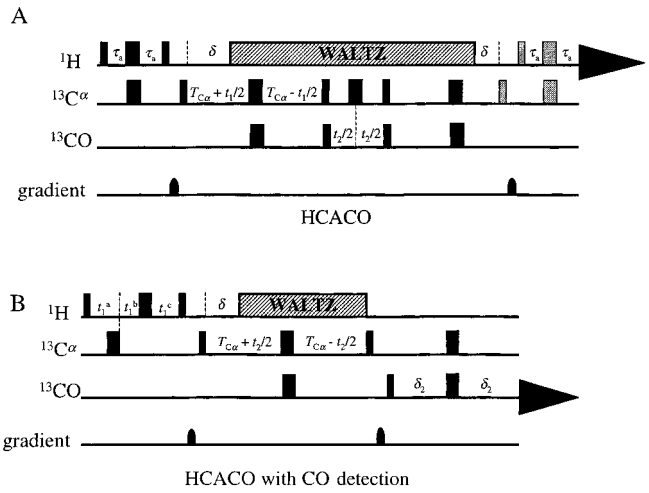

Figure 3. *Comparison of a conventional proton-detected HCACO pulse sequence (A) with a carbonyl-detected version (B).*

detected experiment in Figure 3 B. In the conventional experiment the magnetization is transferred from the α-proton via the α-carbon atom to the carbonyl spin and back through the α-carbon spin to the α-proton for detection during acquisition. This pathway is schematically shown in Figure 4 A. In contrast to this "out-and-back" approach that starts on the α-protons and

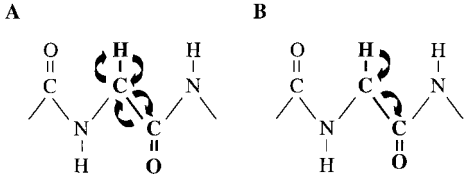

Figure 4. *Schematic diagram of the magnetization transfer pathway for a conventional "out-and-back" HCACO experiment (A) and for a carbon-detected "out-and-stay" pulse sequence (B). Side chains are omitted in the diagram.*

detects on the α-protons, a carbonyl-detected experiment is of the "out-and-stay" type. The magnetization is transferred from the α-proton via the α-carbon atom to the carbonyl group, where it is detected during acquisition (Figure 4B). A comparison of both pulse sequences demonstrates that the carbonyl-detected version is shorter and contains a fewer number of pulses. This minimizes the signal loss due to relaxation and reduces the loss in sensitivity, caused by switching the detection nucleus from proton to carbon. This sensitivity loss is further reduced by the design of the pulse sequence that minimizes the time that the magnetization spends on fast relaxing nuclei, that is, by replacing the fast relaxing α-protons with the slowly relaxing carbonyl spins during acquisition.[3] Figure 5A shows a plane from the carbonyl-detected HCACO experiment. Every peak is split into a doublet by the coupling between the α-carbon atom and the carbonyl group. In Figure 4B the acquisition dimension is not processed with the standard Fourier transformation method, but with a maximum-entropy algorithm.[3, 21] This method is one of several that can be used to deconvolve the coupling without the need for time-shared homonuclear decoupling methods applied during acquisition. A comparison of the one-dimensional slices shown at the top of Figures 5A and B demonstrates that deconvolution of the coupling increases the sensitivity by almost the theoretical factor of two. In Figure 5C, the corresponding plane from a conventional proton-detected HCACO experiment is shown. A comparison of the data shown in Figures 5B and C reveals that the sensitivities of the carbonyl-detected and the proton-detected experiments are very similar demonstrating that a careful design of the pulse sequence can indeed compensate for the original sensitivity loss of a factor of eight. Moreover, in the proton-detected experiment many artifacts around $\delta = 4.8$ in the proton dimension, arising from water suppression, are visible. Since water suppression is not necessary in a carbon-detected experiment, the spectrum in Figure 5B lacks those artifacts completely.

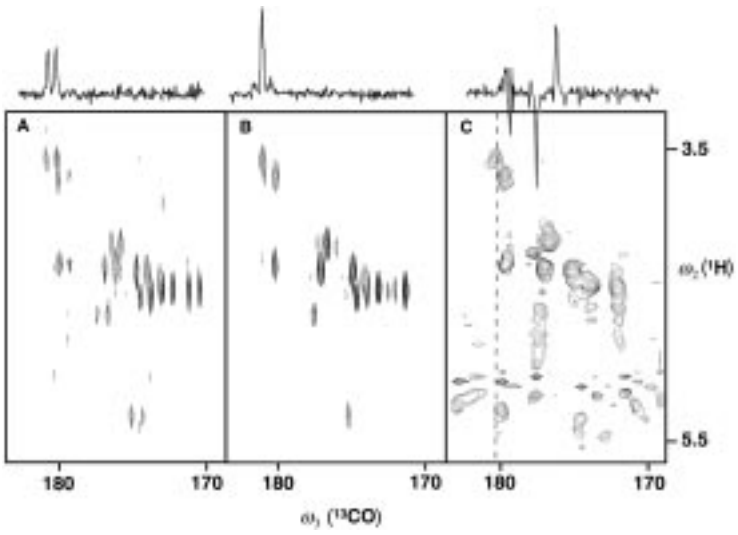

Figure 5. *Section of a $^{1}H\alpha - ^{13}CO$ plane from a three-dimensional HCACO experiment with a 0.6 mM sample of a ^{13}C-labeled 17-kDa fragment of the* E. coli *protein Ada.[26] A: Section taken from a carbonyl-detected HCACO experiment. The carbonyl acquisition dimension was processed by using Fourier transformation, showing the approximately 55-Hz coupling between the $^{13}C\alpha$ and the ^{13}CO spins. B: The acquisition dimension was transformed and the coupling deconvolved by using maximum-entropy reconstruction. C: Section taken from a conventional proton-detected HCACO experiment measured with a triple-resonance cryogenic probe head. The one-dimensional slices on top of each section are taken along the acquisition dimension at the position indicated by the dashed lines (figure reproduced with permission from ref. [3]).*

Applications of carbon-detected experiments

The principle of converting an "out-and-back"- into an "out-and-stay"-type of experiment with the help of carbon detection can also be applied to other NMR experiments. Carbon detection will be particularly useful if carbonyl magnetization can be detected during acquisition due to the slow relaxation of carbonyl spins.[17–19] Moreover, carbonyl spins are only coupled to one additional carbon atom, the α-carbon atom, and this coupling can be suppressed either by homo-decoupling techniques during acquisition or with the help of the deconvolution methods described above. In contrast, most aliphatic carbon atoms have more than one additional carbon atom they are coupled to, producing a more complicated multiplet pattern.

The detection of carbonyl spins during acquisition will also be useful for relaxation experiments that study the dynamics of the protein backbone.[17] With these experiments, one can observe the decay of carbonyl magnetization during a variable delay in the pulse sequence. In the conventional proton-detected experiments, the magnetization is transferred via another heteroatom to a proton for detection. Obviously, in carbonyl-detected experiments, the magnetization can be detected directly after this relaxation delay without any further transfers.

Other applications of carbon detection that we currently study include detection on side-chain carbonyl groups and aromatic carbon atoms for the selective identification of residues with side-chain carbonyl groups and of aromatic amino acids. Carbon detection also provides new methods for the investigation of the structure and dynamics of nucleic acids, which have a smaller number of protons for detection than proteins.

A large number of triple-resonance experiments starts with and detects amide proton magnetization during acquisition. Although many of these experiments can also be modified for carbon detection (e.g. the HNCO or HNCACO experiments), the slower relaxation of the amide group protons relative to the α-protons allows only a smaller compensation of the original loss in sensitivity due to carbon detection. However, at high pH values the amide protons exchange rapidly with protons from the water, leading to fast decay of the signal. This phenomenon, called exchange broadening, can severely reduce the sensitivity of the experiment. Carbon-detected experiments do not suffer from these exchange effects and can, therefore, provide an alternative to NMR experiments at high pH.

In conclusion, carbon detection, now possible with the introduction of cryogenic probe heads, offers new tools for the investigation of biological macromolecules by NMR spectroscopy. It will be particularly useful for

molecules with a low number of protons, such as highly deuterated proteins and nucleic acids, as well as in situations where fast chemical exchange is detrimental to the sensitivity or water suppression problematic.

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