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Assignment of the backbone carbonyl resonances in ^{15}N-labelled proteins with ^{13}C at natural abundance by a 2D triple-resonance correlation technique

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Summary

A 2D NMR experiment for assignment of backbone carbon resonances in small and medium-sized ^{15}N-labelled proteins with ^{13}C at natural abundance is presented. The experiment is a two-dimensional variant of the HNCO triple-resonance experiment and is demonstrated by application to a 6 kDa protein at relatively low concentration (2 mM) and temperature (30 °C). The experiment is particularly suitable for assignment of carbonyl resonances.

The ^{13}C spectrum of a protein provides a variety of information about the dynamical and structural properties of the molecule. First, the ^{13}C relaxation parameters give information about the overall tumbling rates, as well as about internal motions. Second, the ^{13}C chemical shifts of a protein are both sensitive to the local covalent structure and to the local protein conformation. Especially the $^{13}C^{\alpha}$ and backbone carbonyl ^{13}C chemical shifts are sensitive to the backbone conformation and, therefore, contain information about the secondary structure. Third, the ^{13}C spectrum provides a unique set of probes for investigating conformational changes in proteins and interactions between proteins and other biological molecules. In order to take advantage of this diverse information, the ^{13}C spectrum must be assigned so as to allow a correlation between the ^{13}C spectrum and the structure of the protein. Thus, the assignment of the ^{13}C spectrum of a protein is a valuable prerequisite for the characterisation of the dynamical and structural properties of the protein and opens the way for investigations of protein conformational changes and protein interactions.

With the development of two-dimensional ^{1}H-detected ^{1}H-^{13}C-correlated techniques, the potentials for obtaining ^{13}C assignments with ^{13}C at natural abundance have increased dramatically. The inverse one-bond correlation experiments take advantage of the large one-bond scalar coupling between a carbon atom and its directly bound protons, and provide a direct correlation between the ^{13}C chemical shift and the chemical shifts of these protons. Ambiguities caused by resonance overlap in the ^{1}H spectrum can often be overcome by appending a ^{1}H-^{1}H mixing sequence to the basic one-bond correlation experiment (Gao et al., 1990; Kessler et al., 1990). Thus, access to ^{1}H assignments renders the assignment of the proton-bearing carbon atoms of small to medium-sized proteins straightforward (Wagner and Brühwiler, 1986; Gao et al., 1990; Kessler et al., 1990; Kristensen and Led, 1995).

In contrast, the assignment of carbonyl and quarternary carbon resonances cannot be obtained as readily, because the absence of directly bound protons excludes the application of the sensitive one-bond correlation techniques. In proteins with ^{13}C and ^{15}N at natural abundance, one therefore has to rely mainly on multiple-bond correlation techniques. However, the heteronuclear multiple-bond correlation experiment (Bax and Summers, 1986) has not found widespread application to proteins, although in a few favourable cases it has proved useful for obtaining assignments of quarternary carbons (Kessler et al., 1990) and carbonyl carbons (Hansen, 1991).

The assignment of the backbone carbonyl resonances of a protein is of importance for several reasons. First, the carbonyl carbons show a different relaxational behaviour compared to proton-bearing carbons. Where the latter carbons relax primarily through dipole–dipole interactions with the directly bound protons, the chemical shift anisotropy (CSA) mechanism contributes significantly to

the relaxation of carbonyl carbons. It is therefore of interest to see how carbonyl relaxation parameters correlate with the protein structure. Second, Wishart and Sykes (1994) have thoroughly demonstrated that the carbonyl chemical shift is indicative of the local backbone conformation and, hence, the secondary structure. Finally, the backbone carbonyl resonances have been shown to be valuable probes in studies of pH-induced conformational changes in proteins (Abildgaard et al., 1992).

Together with advances in the fields of molecular biology and gene technology, the availability of proteins labelled with ^{13}C and/or ^{15}N has enabled application of a large number of new techniques for obtaining ^{13}C assignments. Markley and co-workers (Westler et al., 1988; Stockman et al., 1989) have developed a set of 2D methods for sequential assignment of the backbone ^{13}C, ^{15}N and ^{1}H resonances in proteins that are partially labelled with ^{13}C in all positions and fully labelled with ^{15}N. These methods take advantage of the relatively large ^{13}C-^{13}C and ^{13}C-^{15}N scalar coupling constants. A related suite of 3D experiments for sequential assignment of backbone resonances of a protein has been developed by Bax and co-workers (Ikura et al., 1990; Kay et al., 1990). These experiments show a superior sensitivity, because they employ both ^{1}H excitation and ^{1}H detection, and can be considered the method of choice for assigning double-labelled proteins.

One of these sequences, the HNCO experiment (Ikura et al., 1990; Kay et al., 1990; Grzesiek and Bax, 1992), correlates the backbone amide proton and nitrogen chemical shifts with the carbonyl chemical shift of the preceding residue. So far, this experiment has, to our knowledge, only been used on $^{13}C/^{15}N$ double-labelled protein samples as part of the general triple-resonance assignment strategy (Ikura et al., 1990).

In this communication we demonstrate the application of a 2D version of the HNCO experiment for obtaining assignments of the carbonyl resonances in proteins that are ^{15}N labelled but have ^{13}C at natural abundance. We show that this strategy for assignment of the backbone carbonyl resonances is applicable to small to medium-sized proteins at relatively low concentration (2 mM) and at moderately low temperature (30 °C). Since the access to $^{15}N/^{13}C$ double-labelled protein samples is still quite limited compared to ^{15}N single-labelled protein samples, we consider this a unique method to obtain carbonyl resonance assignments with ^{13}C at natural abundance.

The pulse sequence of the 2D HNCO experiment, shown in Fig. 1, is analogous to the 3D pulse sequence suggested by Grzesiek and Bax (1992). The pulse sequence involves a transfer of longitudinal amide proton magnetisation to anti-phase ^{15}N magnetisation by means of an INEPT-type magnetisation transfer. The delay 2τ is tuned to $1/(2J_{NH})$. A second INEPT-type magnetisation transfer turns this antiphase magnetisation, into carbonyl transverse magnetisation, which is antiphase with respect to ^{15}N. The delay 2T is tuned to $1/(2J_{NC'})$ or slightly less, in order to reduce the loss in sensitivity caused by relaxation. Simultaneously with this INEPT transfer, the transverse ^{15}N magnetisation is allowed to become in-phase with respect to the amide proton by allowing the ^{15}N magnetisation to evolve with J_{NH} in the delay γ, which is tuned to $1/(2J_{NH})$. The transverse carbonyl magnetisation is now modulated with the chemical shift during the evolution time t_1, after which it is transferred back to the amide proton by means of two consecutive reverse-INEPT magnetisation transfer steps and finally detected as proton magnetisation. Compared with the sequence described by Grzesiek and Bax (1992), we have omitted the constant-time ^{15}N evolution period in order to gain an improvement in signal-to-noise ratio of $\sqrt{2}$. As a result, the ^{15}N dimension of the 3D HNCO experiment collapses

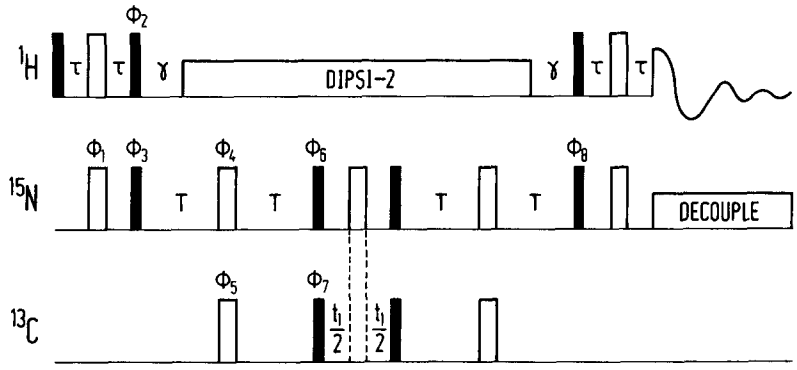

Fig. 1. The proposed pulse sequence, which is analogous to the three-dimensional CT-HNCO experiment proposed by Grzesiek and Bax (1992). Narrow and wide bars represent 90° and 180° pulses, respectively. The phase-cycling scheme is given by $\phi_1 = 4(x,-x)$; $\phi_2 = 4(y,-y)$; $\phi_3 = x$; $\phi_4 = 8(x,-x)$; $\phi_5 = \phi_6 = 2(x,-x)$; $\phi_7 = (x,-x)$ and $\phi_{acq} = (x,-x),2(x,-x),4(x,-x)$. The integral number preceding each bracketed phase element specifies the number of transients acquired before advancing to the next step in the phase element. Quadrature detection was achieved in t_1 by States-TPPI (Marion et al., 1989) of ϕ_3. Note that the last ^{15}N 90° pulse should have phase x, not y as stated by Grzesiek and Bax (1992). The decoupling of ^{1}H, indicated by the wide, low bar, was achieved by a coherent DIPSI-2 modulation (Shaka et al., 1988) of a long low-power ^{1}H pulse. Decoupling of ^{15}N during the acquisition was obtained with a GARP decoupling scheme (Shaka et al., 1985). Delay durations were $\tau = 2.5$ ms, $\gamma = 5.56$ ms and $T = 13.5$ ms.

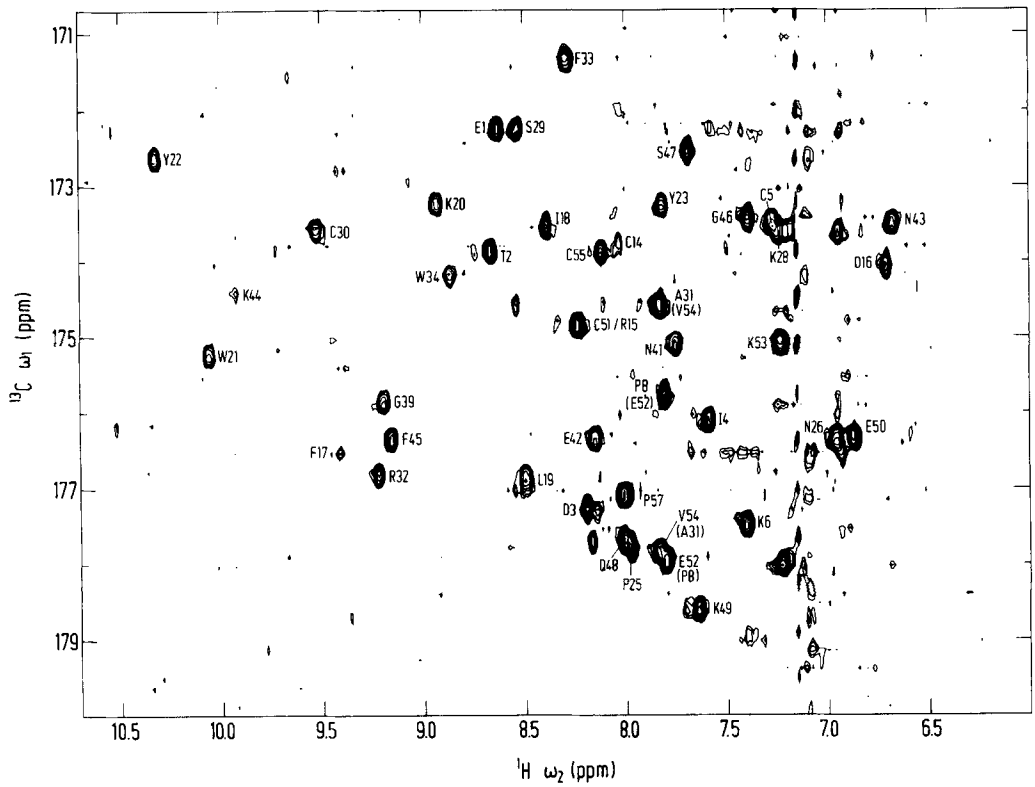

Fig. 2. Contour plot of a 2D HNCO spectrum recorded with the pulse sequence depicted in Fig. 1 on a sample of uniformly (>95) ^{15}N-labelled α3(VI) Kunitz-type domain from human collagen. Only positive contours are shown. The protein concentration was 2 mM, pH 2.9, and the solvent was 90% H_2O / 10% D_2O (v/v). The spectrum was recorded at a sample temperature of 30 °C on a Bruker AM-500 spectrometer operating at ^1H, ^{13}C and ^{15}N frequencies of 500.13, 125.76 and 50.68 MHz, respectively. The spectrometer was equipped with a Bruker [^1H,^{13}C,^{15}N]-triple-resonance probehead and an extra radio-frequency channel for executing the ^{13}C pulses (Kay et al., 1990). The ^1H carrier was positioned on the water resonance at 4.71 ppm in order to allow presaturation of the water during a 1.16 s prescan delay. The coherent DIPSI-2 decoupling (Shaka et al., 1988) of ^1H was applied with a field strength of 5.3 kHz and achieved by a modification of the decoupler circuit, in analogy to the modification described by Mooberry et al. (1994). The ^{15}N carrier was positioned at 118.1 ppm. The ^{15}N pulses and the ^{15}N GARP decoupling (Shaka et al., 1985) were applied with field strengths of 6.6 kHz and 750 Hz, respectively. The ^{13}C carrier was positioned in the carbonyl region at 177.4 ppm and applied with a field strength of 4.0 kHz, giving minimal excitation in the C^α region. The data set consisted of (128 complex) × (3000 real) data points in t_1 and t_2, with corresponding acquisition times of 32 and 150 ms. Four dummy scans were used and 768 scans were accumulated for each t_1 value. Prior to Fourier transformation, the data set was processed with a shifted sine-bell window function and zero-filling to 256 points in t_1, and with a Lorentzian–Gaussian window function and zero-filling to 4096 points in t_2. After the 2D Fourier transform, the spectrum was baseline corrected in ω_2 by an automatic baseline correction procedure analogous to FLATT (Güntert and Wüthrich, 1992). The labels indicate the carbonyl resonances. The carbonyl resonances of residues Pro^8, Ala^{31}, Glu^{52} and Val^{54} are tentatively assigned on the basis of their expected secondary chemical shift.

to give a two-dimensional ^1H-^{13}C-correlated HNCO experiment. Also, we have omitted the fourth-channel carbon refocusing pulses because they are redundant for proteins with ^{13}C at natural abundance.

Figure 2 shows the 2D HNCO spectrum of the α3(VI) Kunitz-type domain from human collagen, recorded with the pulse sequence of Fig. 1. The spectrum in Fig. 2 displays a substantial number of correlations with intensities well above the noise level. The α3(VI) Kunitz-type domain from human collagen is a 58-residue globular domain with three prolines. Ideally, we would therefore expect 54 correlations in the HNCO spectrum when counting out the prolines and the C-terminal amino acid residue. By careful examination, it is possible to observe 42 correlations in the spectrum. By comparison with a ^{15}N-edited TOCSY spectrum recorded under exactly the same experimental conditions, and by using the amide proton assignments obtained by standard 2D methods, it is possible to sequentially assign 37 of these correlations. A few correlations could not be assigned unambiguously because of resonance overlap in the amide proton region. One way to resolve this overlap would be to record a spectrum at a different temperature or a different pH. An alternative method would be to include the constant-time ^{15}N evolution period to separate the correlations in a third dimension by means of the ^{15}N chemical shift; however, at the expense of the sensitivity of the experiment.

The chemical-shift indices of the α3(VI) carbonyl resonances, as defined by Wishart and Sykes (1994), are shown in Fig. 3 together with information about secondary structure derived from NOEs (Sørensen, M.D. et al., manuscript in preparation). As can be seen from the

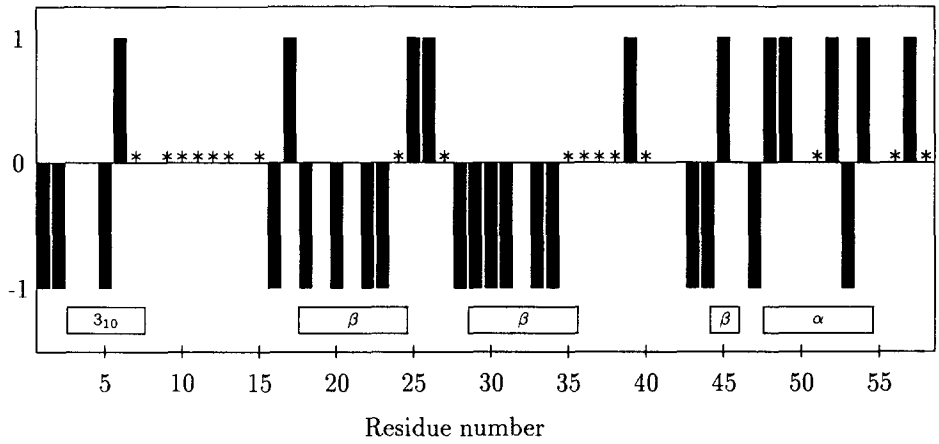

Fig. 3. The carbonyl ^{13}C chemical shift indices calculated according to Wishart and Sykes (1994). The ^{13}C chemical shifts were measured relative to external dioxane at 69.28 ppm. The carbonyl resonances of the residues marked with an asterisk are unassigned. Also shown is the secondary structure determined from NOEs.

figure, good agreement exists in general between the chemical-shift index and the secondary structure derived from the NOEs.

The basic pulse sequence depicted in Fig. 1 could probably gain considerably by applying pulsed field gradients for either suppression of artefacts or coherence selection. Especially the water suppression could be improved considerably by applying a gradient-based pulse scheme. Furthermore, the pulse sequence in Fig. 1 could equally well be used to record an HNCA-type spectrum by positioning the carbon transmitter in the $^{13}C^{\alpha}$-region or, alternatively, it could be used to record a combined HNCA and HNCO spectrum by applying high-power ^{13}C pulses and positioning the carbon transmitter in the centre of the ^{13}C spectrum. However, because of the smaller $J_{NC\alpha}$ coupling constant and the faster relaxation rate of the α-carbon, the correlations to the α-carbons are expected to be less intense.

In conclusion, we have shown that a two-dimensional variant of the HNCO experiment can be used to obtain backbone carbonyl resonance assignments of ^{15}N-labelled proteins with ^{13}C at natural abundance. Furthermore, we have demonstrated that it is feasible to perform this experiment on a 2 mM sample of the 58-residue α3(VI) Kunitz-type domain from human collagen at 30 °C, i.e., at relatively low concentration and temperature. Thus, the technique should find widespread application for the assignment of backbone carbonyl resonances in small and medium-sized ^{15}N-labelled proteins.

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