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Design of small volume HX and triple-resonance probes for improved limits of detection in protein NMR experiments

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Abstract

Three- and four-frequency nuclear magnetic-resonance probes have been designed for the study of small amounts of protein. Both "HX" (^{1}H, X, and ^{2}H channels) and "triple-resonance" (^{1}H, ^{15}N, ^{13}C, and ^{2}H) probes were implemented using a single transmit/receive coil and multiple-frequency impedance matching circuits. The coil used was a six-turn solenoid with an observe volume of 15 μl. A variable pitch design was used to improve the B_1 homogeneity of the coil. Two-dimensional HSQC spectra of ~1 mM single labeled ^{15}N- and double labeled ^{15}N/^{13}C-proteins were acquired in experimental times of approximately 2 h. Triple-resonance capability of the small-volume triple-resonance probe was demonstrated by acquiring three-dimensional HNCO spectra from the same protein samples. In addition to enabling very small quantities of protein to be used, the extremely short pulse widths (^{1}H = 4, ^{15}N = 4, and ^{13}C = 2 μs) of this particular design result in low power decoupling and wide-bandwidth coverage, an important factor for the ever-higher operating frequencies used for protein NMR studies.

1. Introduction

One of the most important applications of solution-state NMR is the study of protein structure and dynamics. The sophisticated multi-dimensional experiments needed for such studies require that the protein be partially or fully isotopically labeled, with either single or multiple labels. In comparison to other analytical techniques, relatively large amounts of protein are required for these studies, typically hundreds of nanomoles. The two "controllable" factors that affect the sensitivity of the particular NMR experiment are the strength of the main magnetic field and the sensitivity of the NMR probe. There are many reasons why it is desirable to improve the probe performance. These include the ability to study proteins which aggregate at relatively low concentrations, the use of smaller amounts of labeled proteins which can be expensive and/or time-intensive to prepare, faster throughput in applications such as structure–activity relationships, e.g., SAR by NMR [1], and improved performance in terms of decoupling bandwidths and powers at very high operating frequencies. Finally, the improvement in NMR performance from probe technology is generally much less expensive than comparable gains from magnet technology.

Most commercial probes designed for protein studies use a "saddle" and modified Alderman–Grant [2] geometries for the inner and outer radiofrequency (RF) coils, respectively, in the probe. The inner coil is usually tuned to both proton and deuterium frequencies, with the impedance matching circuits designed to optimize the sensitivity of the proton channel. The outer coil has a larger diameter than, and is rotated by 90° with respect

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to, the inner coil. This outer coil is impedance matched to 50 Ω at two or more frequencies, usually ^{15}N and ^{13}C. An advantage of this two-coil setup is that the sensitivity of the proton channel is not affected significantly by the presence of the other channels. Another attractive feature of the conventional vertical coil orientation is the convenience of simple sample changing. An important disadvantage of this coil geometry is that the coupling between the sample and the RF coil for the heteronuclear channels is relatively weak due to the low filling factor. This poor coupling typically results in long pulse widths on the carbon and, in particular, the nitrogen channel. This is a particular concern at very high magnetic fields, where effective heteronuclear decoupling over a large bandwidth becomes problematic.

The vertical geometry uses NMR tubes that are designed to minimize magnetic-susceptibility mismatches around the liquid samples by approximating an infinite, vertically oriented cylinder of liquid extending above and below the RF coil. Practically, at least one-third of the sample must lie both above and below the coil in order for this to be a valid approximation, and for shimming to be effective. This requirement results in an observe factor, defined as the ratio of the sample volume contained within the coil to the total sample volume, of approximately 33%. The use of susceptibility-matched plugs [3] to reduce sample volumes without significantly affecting the B_0 homogeneity has found some use in protein NMR studies in cases where the sample is mass-limited. Typically this approach, in which cylindrical plugs made from material of very similar magnetic susceptibility to the solute are placed below and above the actual sample, increases the observe factor from 33 to above 80%: the loss in spectroscopic resolution is minor but shimming usually requires more time and effort.

Recent advances in cryogenics have resulted in the design of NMR "cryoprobes" [4]. The essential geometry of these probes is very similar to that described above, with the key difference being that the operating temperature of the sample coil and the preamplifier are reduced to a few degrees Kelvin using circulating cold helium gas, such that the noise contribution from the coil is significantly reduced. The coils can either be made from copper, thin-film superconducting materials such as yttrium barium copper oxide (YBCO), or aluminum. Despite requiring coils of larger diameters than those operating at room-temperature, thus reducing the filling factor, the signal-to-noise ratio (SNR) using cryoprobes increases by a factor of four in comparison to conventional probes [5] for non-lossy samples. An alternative approach to increase the intrinsic mass sensitivity, albeit at the expense of the concentration sensitivity, is to reduce the coil dimensions. The smallest probe with a saddle-geometry coil is approximately 1.3 mm in diameter [6], with an active volume of 5 μl, which can be used with sample tubes up to 1 mm in diameter. This reduction in size increases the mass sensitivity of the probe by a factor of four to five compared with a 5 mm probe, but this particular sample volume is too small for studies of proteins at reasonable concentrations.

Although the vertically oriented coil design has many practical advantages, it is well known that a solenoidal coil has a much higher intrinsic sensitivity due to stronger coupling to the sample [7]. Despite the higher sensitivity, the use of solenoidal coils in high-resolution liquid-state NMR has only become routine relatively recently. The major implementation issue has been achieving the necessary B_0 homogeneity throughout the sample. Two approaches to B_0 homogeneity optimization have been taken. In the first, a solenoid (approximately 4 mm in diameter) constructed of magnetic-susceptibility matched wire is oriented at the magic angle with respect to the B_0 axis. The sample is introduced into either a spherical or cylindrical container and spun at speeds of a few kHz. Spinning at the magic angle reduces the effect of magnetic-susceptibility discontinuities by up to two to three orders of magnitude for a cylindrically symmetric geometry, and allows high resolution spectra to be obtained from a coil observe factor of essentially 100%. However, orientation at the magic angle intrinsically involves a loss in sensitivity of a factor of approximately 20% compared to a horizontally oriented solenoid. The sample volume using this probe may be reduced to as small as 40 μl [8–10]. Investigations of small quantities of natural products including peptides and small proteins using this approach have been reported for proton-only probes [11] and also using a commercially available gradient-HX version [12], but very limited protein work has been performed using HRMAS probes, probably due to several factors including requirements for additional spinning hardware, the unavoidable presence of spinning side bands, difficulties in sample handling and loading, and the historical lack of triple-resonance capabilities.

The second approach to obtaining high-resolution spectra using solenoidal coils has been the use of a perfluorinated susceptibility-matching fluid [13,14]. With this approach, sub-millimeter coils have been used for a variety of applications with coil observe volumes ranging from 5 nl to approximately 1 μl [15]. In this paper we demonstrate efficient multiple-frequency designs, in combination with a simple variable-pitch solenoidal coil, that reduce the mass of protein in NMR experiments by almost an order of magnitude compared to a 5 mm probe, without cryogenic or magic angle spinning approaches. The observe volume of the coil has been increased with respect to previously reported "microcoils" to approximately 15 μl so that realistic concentrations of proteins can be studied in reasonable data acquisition times. The solenoidal design results in extremely short pulse widths on all three channels. These

coils can produce high quality 2D spectra from proteins at a concentration of ~1 mM in 2 h or less, and require 45 nmol of sample in total (~20 nmol if susceptibility-matched plugs are not used). Three-dimensional triple-resonance data from the same amount of sample are also easily obtained.

2. Probe design

Two types of NMR probe were designed: the first was a three-channel, "HX" probe consisting of ^{1}H, ^{15}N, and ^{2}H lock channels. The second was a four-channel, "triple-resonance" design, consisting of ^{1}H, ^{13}C, ^{15}N, and ^{2}H lock. A triple-axis gradient set, with a clear bore of 20 mm, was installed on the HX probe. A commercial probe body (Magnetic Resonance Microsensors Corporation, Savoy, IL) was used to house the probe assembly.

The sample coils in the two probes use the same design. A six-turn solenoid with diameter 2.5 mm and length 4 mm was fabricated using 26 AWG (405 μm diameter) copper wire (California Fine Wire, Grover Beach, CA). The coil was wound on a Pyrex capillary (Wilmad Glass, Buena, NJ) with an inner diameter of 2.2 mm. The observe volume of the coil is 15 μl. A variable pitch design is used to improve the B_1 homogeneity of the solenoid [16,17]. The inter-turn spacings from the center to the two sides of the coil are 405, 320, and 255 μm, respectively. The variable pitch design improves the B_1 homogeneity significantly with a small decrease in the sensitivity due to the increase of the interturn space at the center of the coil. The design was implemented starting from a uniformly double-wound coil using 405 μm (AWG 26) and 255 μm (AWG 30) diameter wire. The 255 μm wire was then unwound, and 405 and 320 μm (AWG 28) wires were used to increase the interturn spacing between the central two turns and the other turns. The 405 μm wire is stiff enough to keep the windings tight. Susceptibility matching fluid (FC-43, 3M Corp., St. Paul, MN) was placed around the solenoid coil to improve the B_0 homogeneity, as described in previous studies [13,14]. The sample was inserted into the capillary tube upon which the coil was wound. Due to space constrictions, and the requirement for high B_0 homogeneity, the leads from the coil to the matching networks are relatively long, ~2.5 cm, and this leads to significant losses in the overall probe design. Designs to overcome this shortcoming are currently being investigated.

The impedance matching networks in the two probes used a single-coil/multiple-resonance RF design. A variety of designs exist for multiple-tuned circuits and different approaches have formed the basis of several short review articles [18–21]. Many implementations have been presented for tuning a coil to two frequencies for both spectroscopy and imaging applications: these include multipole [22–25], overcoupling [26–28], and a variety of other methods [29–35]. Designs for tuning a single coil to three or four frequencies have also been implemented [36–41].

Since LC trap circuits are used to separate channels, circuit losses arise mainly from these traps. Extensive modeling (ADS, Hewlett-Packard, Santa Rosa, CA) of different impedance matching network designs was carried out in order to optimize the sensitivity of the probe, in particular the proton channel, and to maximize the isolation between different frequency channels. As an example of the utility of computer modeling, the low frequency series LC tank is important for the proton channel sensitivity, but it also plays a critical role in the sensivitity and tunability of the low frequency channels. A large inductance increases the proton sensitivity but causes low sensitivity and difficulties in tuning the other

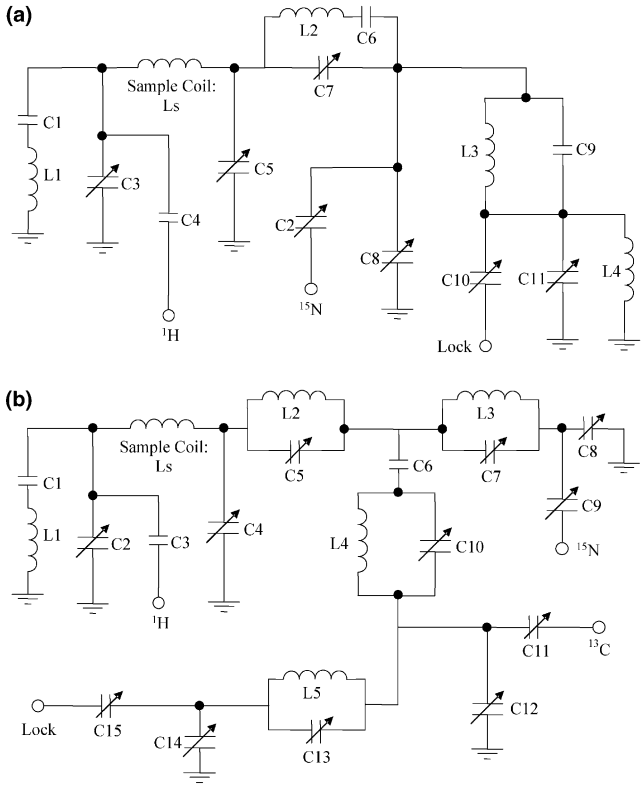

Fig. 1. (a) RF circuit for the three-channel, "HX," probe. Variable capacitors are either single 0.5–8 pF units, or are in parallel with a fixed capacitor. Component values: C1 = 200 pF, C2 = 8.7–16.2 pF, C3 = 0.5–8 pF, C4 = 0.5 pF, C5 = 0.5–8 pF, C6 = 188 pF, C7 = 0.5–8 pF, C8 = 50.5–58 pF, C9 = 82 pF, C10 = 7.3–14.8 pF, C11 = 12.5–20 pF, L1 = 34 nH, L2 = 36 nH, L3 = 84 nH, and L4 = 66 nH. (b) RF circuit for the four-channel, "triple-resonance," probe. Component values: C1 = 82 pF, C2 = 0.5–8 pF, C3 = 0.3–1.2 pF, C4 = 0.5–8 pF, C5 = 4.8–8.3 pF, C6 = 56 pF, C7 = 8.2 pF, C8 = 39.5–47 pF, C9 = 3.8–11.3 pF, C10 = 8.7–16.2 pF, C11 = 0.5–8 pF, C12 = 0.5–8 pF, C13 = 6.1–13.6 pF, C14 = 39.5–47 pF, C15 = 12.5–20 pF, L1 = 34 nH, L2 = 32 nH, L3 = 108 nH, L4 = 345 nH, and L5 = 121 nH.

channels. Therefore, a compromise between these two factors has to be incorporated. The circuit implementation used both fixed-value capacitors (American Technical Ceramics, Huntington Station, NY) and variable capacitors (Voltronics Corporation, Denville NJ).

The RF circuit for the HX probe is shown in Fig. 1a. The L1-C1 trap presents a very small impedance at low frequencies and large impedance at high frequencies. A high quality (Q) factor L2-C6-C7 tank results in a high impedance at the proton frequency and a very low impedance path at the ^{15}N frequency. Thus, balanced matching is achieved for the proton channel, but matching for the other channels is at least partially unbalanced. The proton channel has the shortest electrical path to the sample coil in order to minimize signal loss. In terms of efficiency, the least important channel is the lock channel, for which inductive matching was necessary since the lock frequency is higher than the ^{15}N frequency. However, since the sample volume is small, the lock channel cannot be extremely insensitive, unlike the case for much larger coils. A parallel LC (L3-C9) trap, resonant at the ^{15}N frequency, is used between the ^{15}N channel and lock channel. The RF circuit used for the triple-resonance probe is shown in Fig. 1b. Differences from the HX circuit are that LC trap circuits at both ^{15}N and ^{13}C frequencies are used between the ^{15}N and ^{13}C channels, and the lock channel is attached to the ^{13}C channel with a trap circuit at the ^{13}C frequency. Since the ^{13}C frequency is higher than the lock frequency, capacitive matching can be used.

3. Experimental

NMR data were obtained on two different 600 MHz spectrometers, a Varian Inova at the University of Illinois and a Bruker Avance at the University of Florida. Both spectrometers were equipped with Oxford Instruments 14.1 T standard (51 mm) bore magnets. A commercial 5 mm triple-resonance probe (TRIAX 0190159200, Varian, Palo Alto, CA) was used for comparative studies. Two-dimensional heteronuclear single quantum coherence (HSQC) data were collected on both systems, and 3D HNCO data were collected on the Bruker system. Solvent suppression on the HX probe was performed using a WET sequence [42,43]. Solvent suppression on the triple-resonance probe was performed with presaturation, and non-gradient-based pulse sequences were used for data acquisition. Data were processed with NMRPipe [44] by first minimizing the residual water signal by deconvolution, multiplying the data in both dimensions by cosine squared functions, zero-filling once, Fourier transformation, and baseline correction. Data were analyzed with the NMRView software package [45].

Two different proteins were used in this study. IA-3 is a 68 amino acid endogenous inhibitor of yeast proteinase A and is an unstructured random-coil protein when not bound to YprA [46]. IA-3 samples were 1 mM, ^{15}N-labeled for HSQC or $\{^{15}N + ^{13}C\}$-labeled for the HNCO experiments, and were prepared in 50 mM phosphate buffer, 90% H_2O/10% D_2O at a pH of 5.5. The second protein, ^{15}N-labeled ubiquitin (VLI research, Malvern, PA), was used at a concentration of 1.25 mM in 50 mM phosphate buffer, 95% H_2O/5% D_2O at a pH of 5.8.

4. Results

The electrical characteristics of the HX probe are shown in Table 1. The proton channel has the highest Q value, and the lock channel has the lowest Q value due to the use of inductive matching. A very high degree of electrical isolation, necessary for high sensitivity proton detection, exists between the proton channel and the other two channels. The isolation between the ^{15}N and the lock channels at the lock frequency is not very high. However, the experimental setup involves placing an external filter in the cables connected to the lock channel. This filter has a high impedance at the ^{15}N frequency, providing an additional 30 dB of isolation. Electrical characteristics of the triple-resonance probe are shown in Table 2.

A 1% H_2O/99% D_2O sample was used to measure the B_1 homogeneity of the RF coil. The 810°/90° ratio of the coil increased from 40% for a uniform-spaced design to 60% with variable spacing (data not shown). The 90° pulse widths for all channels are shown in Table 3, together with those from the commercial 5 mm triple-resonance probe for comparison. As expected, the pulse widths are considerably shorter for the solenoidal coils, reflecting the increases in sensitivity from a more efficient intrinsic design and also a reduced diameter. In particular, the 90° pulse widths for the ^{15}N and ^{13}C channels decrease dramatically compared to the 5 mm probe.

Table 1
Electrical characterization of the HX solenoid probe

Electrical parameters		Without sample	With sample[a]
Q (^1H channel)		151	91
Q (^{15}N channel)		50	42
Q (^2H channel)		24	20
S_{21} $^1H/^{15}N$	600 MHz	−45 dB	−45 dB
	60.8 MHz	−54 dB	−51 dB
S_{21} $^1H/^2H$	600 MHz	−54 dB	−54 dB
	92.1 MHz	−58 dB	−56 dB
S_{21} $^{15}N/^2H$	92.1 MHz	−5 dB	−5 dB
	60.8 MHz	−47 dB	−46 dB

[a] The sample was 1 mM IA-3 in 50 mM phosphate buffer.

Table 2
Electrical characterization of the triple-resonance solenoid NMR probe

Electrical parameters		Without sample	With sample[a]
Q (^{1}H channel)		142	95
Q (^{13}C channel)		95	81
Q (^{15}N channel)		65	58
Q (^{2}H channel)		56	51
S_{21} ^{1}H/^{13}C	600 MHz	−50 dB	−54 dB
	150.9 MHz	−39 dB	−38 dB
S_{21} ^{1}H/^{15}N	600 MHz	−53 dB	−54 dB
	60.8 MHz	−34 dB	−36 dB
S_{21} ^{1}H/^{2}H	600 MHz	−68 dB	−69 dB
	92.1 MHz	−53 dB	−51 dB
S_{21} ^{13}C/^{15}N	150.9 MHz	−46 dB	−48 dB
	60.8 MHz	−74 dB	−73 dB
S_{21} ^{13}C/^{2}H	150.9 MHz	−44 dB	−48 dB
	92.1 MHz	−8 dB	−8 dB
S_{21} ^{15}N/^{2}H	92.1 MHz	−20 dB	−18 dB
	60.8 MHz	−63 dB	−63 dB

[a] The sample was 1 mM IA-3 in 50 mM phosphate buffer.

Table 3
Pulse widths for the solenoid and 5 mm triple-resonance TXI probes

Probe	90° Pulse width[a]		
	^{1}H[b] (μs)	^{15}N[c] (μs)	^{13}C[c] (μs)
HX solenoid	4.8	4.1	
Triple-resonance solenoid	4.0	3.8	1.8
5 mm Triple-resonance TXI probe	12	43	14

[a] The sample in the pulse calibration experiment is 1 mM *N*-acetylglycine in 90% H_2O/10% D_2O, pH 5.5.
[b] The proton amplifier has a maximum output of 50 W; the pulses are measured using 6 dB of attenuation.
[c] The amplifier for the ^{15}N and ^{13}C channels has a maximum output of 300 W; the pulses are measured at full power.

A ^{1}H–{^{15}N} gradient HSQC spectrum of ubiquitin acquired with the HX solenoidal probe is shown in Fig. 2. In this probe, a total protein sample volume of 45 μl was used. A previous investigation using solenoidal coils suggested that an observe factor of 70% could be achieved by using perfluorocarbon plugs to limit the sample volume without any degradation in spectral resolution when compared to an infinite cylinder of sample [47]. This would reduce the required sample volume to approximately 20 μl. In these experiments, however, we have used a conservative value of 33% for the observe factor by including 15 μl of sample either side of the coil. This makes for simple comparisons to the commercial 5 mm probe, since this also has an observe factor of approximately 33%. For both the HX and triple-resonance solenoidal probes the full width at half-maximum (FWHM) value for the water peak was ~13 Hz: shimming was performed using the lock signal.

Fig. 3 shows a sensitivity comparison between the HX solenoidal probe and the commercial 5 mm probe.

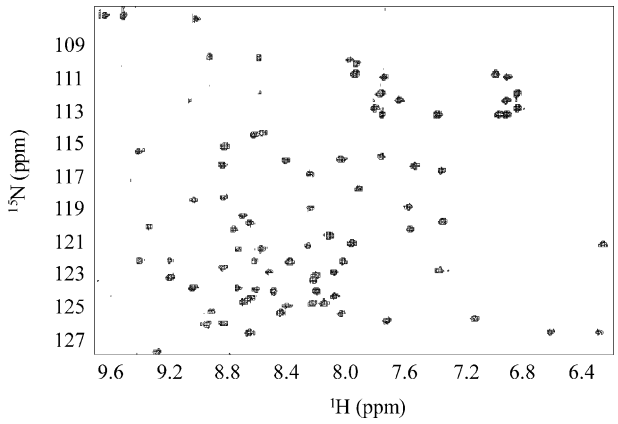

Fig. 2. A gradient ^{1}H–{^{15}N} HSQC spectrum of ubiquitin (1.25 mM) using the HX solenoidal probe. Experimental parameters: sw 3000 Hz, sw1 1300 Hz, 32 signal averages, 96 × 2 hypercomplex increments, 1712 complex data points, relaxation delay 1.5 s, total data acquisition time 2 h.

The same sample mass was used in the two ^{1}H–{^{15}N} HSQC experiments. In the solenoidal probe 60 μl of 1 mM ^{15}N-labeled IA-3 was used, whereas in the 5 mm probe 600 μl of 0.1 mM IA-3 was used. Both datasets were collected with the same acquisition parameters and processed identically. The SNR of the spectra acquired using the solenoidal coil is approximately ten times greater than that of the 5 mm probe.

Fig. 4 shows 3D HNCO spectra collected on a 1 mM, double-labeled ^{15}N/^{13}C IA-3 using the triple-resonance

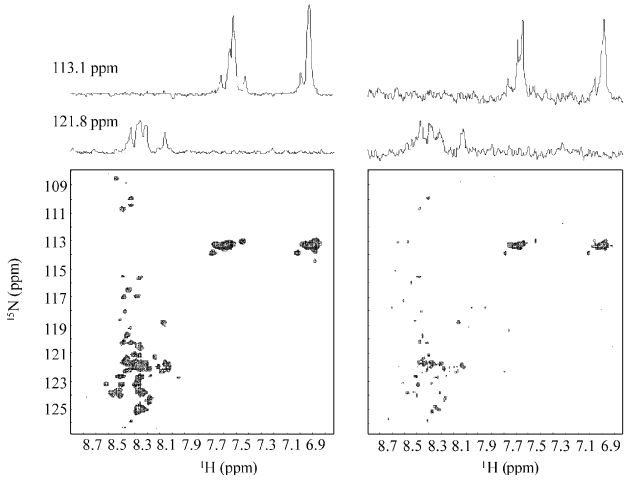

Fig. 3. A sensitivity comparison of the HX solenoidal probe and a commercial 5 mm probe using equal amounts of ^{15}N-labeled IA-3: the data were acquired using a ^{1}H–{^{15}N} HSQC sequence. Experimental parameters were as described in Fig. 2. The solenoidal probe contained 60 μl of 1 mM ^{15}N-labeled IA-3, and the 5 mm probe contained 600 μl at a concentration of 0.1 mM. Two 1-D slices at 113.1 and 121.8 ppm in the ^{15}N dimension are shown on top of the 2D spectrum: spectra from the 5 mm probe are shown at a 10-times higher scale. The signal-to-noise ratios of the maximum peaks in these slices are 130:1 and 13:1 (113.1 ppm) and 60:1 and 6.5:1 (121.8 ppm).

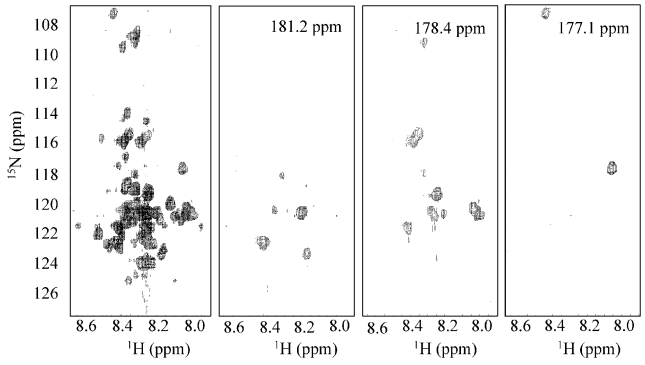

Fig. 4. Data from a 3D HNCO spectrum of double-labeled 1 mM ^{15}N/^{13}C IA-3 (45 μl) using the triple-resonance solenoidal probe. Experimental parameters: sw 6614 Hz, sw1 (^{15}N) 1302 Hz, sw2 (^{13}C) 3001 Hz, 32 signal averages, 64 real data points in the ^{13}C═O dimension, 60 real data points in the ^{15}N dimension, 4096 complex acquisition data points, total data acquisition time 59 h. Solvent suppression used presaturation. The 2D projection of all the ^{13}C═O frequencies is shown in the leftmost panel, and the plots of selected single ^{13}C═O slices are shown in the other three panels.

solenoidal probe. The left panel of Fig. 4 shows a 2D projection of the ^{1}H, ^{15}N correlations, and the three remaining panels show specific 2D ^{1}H, ^{15}N planes extracted at different ^{13}C═O frequencies.

5. Discussion

Although many protein investigations can be carried out using existing commercial NMR probes, there is a strong motivation for continuing to increase detector efficiency. In practical terms this corresponds to optimization for the smallest possible sample mass, and to enable the shortest pulse widths and lowest decoupling power to be used. It has been shown here that small solenoidal coils can be used to acquire HSQC spectra of proteins at 1 mM concentration in a couple of hours. Lower concentrations can clearly also be investigated with longer data acquisition times or higher field magnets. An HNCO experiment using 45 nmol of protein sample proved the multi-dimensional triple-resonance capability of this type of small-volume probe. Increases in sensitivity, compared to a commercial 5 mm probe, are at least comparable to those reported for cryoprobes. For studies of proteins that can only exist in solution at very low concentration, the optimal approach is undoubtedly to use cryogenic technology in the design of coil and preamplifier. However, this approach effectively restricts the experimentalist to the use of commercial probes, with limited capacity for a tailored approach for specific projects. Also, the nature of the cryoprobe means that a magnet must effectively be dedicated solely to the use of this one probe. The circuit designs presented in this paper should allow investigators to construct their own high sensitivity probes and are easily adapted for different field-strengths, with the addition of extra frequency channels if required.

In terms of the specific design of a multiple-frequency probe for protein samples, a number of compromises are necessarily involved. The sample coil is required to be resonant at four different frequencies, with a factor of 10 between the highest and lowest frequency. It is not possible to obtain a high Q value for each channel over this large frequency range, and the matching networks must be designed to optimize the ^{1}H sensitivity, but also with the aim of maintaining reasonable sensitivity on the ^{15}N and ^{13}C channels so that short pulses can be used. The dimensions of the coil also involve some trade-offs, particularly at high frequencies where the intrinsically low self-resonant frequency of the solenoidal geometry imposes restrictions on the number of turns that can be used. The observe volume, length and diameter of the coil, wire thickness and wire spacing are all parameters that have to be considered. Many of these factors are discussed thoroughly in references [48] and [49].

Finally, the reduction in size of the NMR coil has another potential advantage: the possibility of designing probeheads with more than one coil, allowing parallel rather than serial data acquisition to be performed. Various schemes to this end have already been proposed [50–52]. The extension of the multi-coil approach to full multi-frequency capability necessary for running multiple protein samples simultaneously is not simple, but is a goal worth pursuing.

Acknowledgments

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