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Biomolecular NMR Using a Microcoil NMR Probe − New Technique for the Chemical Shift Assignment of Aromatic Side Chains in Proteins

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Abstract: A specially designed microcoil probe for use in biomolecular NMR spectroscopy is presented. The microcoil probe shows a mass-based sensitivity increase of a minimal factor of 7.5, allowing for the first time routine biomolecular NMR spectroscopy with microgram amounts of proteins. In addition, the exceptional radio frequency capabilities of this probe allowed us to record an aliphatic−aromatic HCCH-TOCSY spectrum for the first time. Using this spectrum, the side chains of aliphatic and aromatic amino acids can be completely assigned using only a single experiment. Using the conserved hypothetical protein TM0979 from *Thermotoga maritima*, we demonstrate the capabilities of this microcoil NMR probe to completely pursue the sequence specific backbone assignment with less than 500 μg of ^{13}C,^{15}N labeled protein.

Introduction

NMR spectroscopy is a well-established tool for the structure determination of proteins and nucleic acids.[1] Using uniformly $^{15}N/^{13}C$ labeled protein, NMR has proven not only to be a valuable tool for structure determination but also provides a wealth of information on protein dynamics,[2] as well as information about binding partners, that is, small molecule ligands[3] or biomacromolecules.[4]

It is a well-known fact that NMR spectroscopy suffers from rather low sensitivity and thus requires considerable amounts of protein for these aforementioned experimental measurements. Preparation of these large amounts of protein is expensive (^{15}N,^{13}C labeling), and moreover not all proteins can be expressed in large enough yields. To reduce the amounts of protein required for reasonable measurement times, one of the main goals for technical development in NMR spectroscopy has always been to increase the sensitivity of the NMR instrumentation. In a review of the parameters that affect the sensitivity of the NMR measurement, the five most important are as follows: (1) the static magnetic field (the signal-to-noise ratio is proportional to $B_0^{3/2}$, highest field commercially available: 21.2 T), (2) the sample concentration (for NMR studies, protein concentrations of 0.5 mM or more are needed), (3) the electronic noise of the spectrometer and probe,[5,6] (4) the coil sensitivity,[7] and (5) new pulse sequence methods that increase the sensitivity by using distinctive magnetization transfer pathways.[8,9] In the past few years, the introduction of cryoprobe technology,[5,6,10] where the preamplifier and the Rf-coils are cooled to ∼30 K, has been the most promising technology in reducing the electronic probe noise, thus increasing the signal sensitivity on average by a factor of 3. However, even in using cryoprobes, one still uses standard 5 mm NMR tubes where the sample volume needs to be a minimum of 500 μL to achieve homogeneity. This requirement still demands considerable amounts of protein to maintain reasonable sample concentrations for high-quality measurements. Also, for rather dilute samples, the water suppression can be difficult when using a cryoprobe. Newly developed 3 mm microcryoprobes with total volumes of approximately 110 μL are available, but, as reported, water suppression is not easily accomplished with theses probes, a major drawback for biomolecular NMR.

Another approach in increasing the probe sensitivity has come from microcoil probe technology.[11,12] The increase in the signal-to-noise of microcoils with a fixed length-to-diameter ratio

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improves as the coil diameter decreases;[7,11,13,14] that is, the sensitivity of the NMR measurement is, to a first approximation, inversely proportional to the coil diameter[7] ($\sim 1/d$). To achieve a balance between mass sensitivity and sample load, commercially available microcoil NMR probes utilize 1 mm inner diameter coils with 3 mm length. The total flow cell volume of these microcoils is a scant 5 μL, having an active NMR volume of 1.5 μL. This volume is approximately 180 times less than that for standard 5 mm probes.

While microcoil NMR probes have been available commercially for several years for small molecule work,[15] their use for biomolecular NMR data acquisition has never been exploited until recently.[14] Here, we demonstrate the use of a new microcoil flow-through NMR probe, the CapNMR probe (MRM Corp., Savoy, USA), for the recording of heteronuclear triple resonance NMR spectroscopic data similar to those measured using a traditional 5 mm NMR probe. With only microgram amounts of protein, we have been able to acquire a full set of data for the complete sequential assignment of a protein.

Taking advantage of the excellent radio frequency capabilities of this probe, which are a result of the solenoid coil design, we have demonstrated for the first time the TOCSY transfer between the aliphatic−aromatic region in a HCCH-TOCSY spectrum.[16,17] This experiment now allows for the complete side-chain assignment of all amino acids in a protein within a single spectrum. The obstacle for this aliphatic−aromatic carbon transfer has always been the large carbon chemical shift differences between aliphatic and aromatic residues (average ~13 200 Hz at 14.1 T), creating a requirement for high Rf-power demand necessary to establish a full TOCSY transfer across these aliphatic−aromatic residues. This broad TOCSY transfer capability could not be achieved using traditional 5 mm room temperature or cryoprobes. The success of this experiment has allowed for the aromatic side-chain assignment, including connectivities with the aliphatic side chains, to be possible in a single NMR experiment. This greatly accelerates the aromatic side-chain assignment.

Materials

Most of the experiments presented in this paper were performed using proteins from the *Thermotoga maritima* proteome, in particular, the conserved hypothetical protein, TM0979[18] (PDB id: 1RHX, BMRB id: 6010, 87 residues, 9.7 kDa). For initial sensitivity tests, commercially available BPTI (bovine pancreatic trypsin inhibitor, Bayer AG, Germany) was used (0.24−15.38 mM samples, 20 mM Na phosphate buffer pH 6.0, 10% D_2O). Between 2 and 10 mM samples of either ^{15}N or ^{15}N,^{13}C labeled TM0979 (20 mM Na phosphate buffer pH 6.0, 50 mM NaCl, 10% D_2O, 0.3% NaN_3) were used to record various heteronuclear triple resonance spectra. The ^{15}N labeled sample consisted of 29.6 μg of protein in the active volume, while 148.1 μg of protein for the $^{15}N/^{13}C$ labeled sample was used. The measurements were performed between 293 and 313 K.

Methods

2D homo- and heteronuclear correlation and 3D heteronuclear triple resonance spectra have been recorded[19,20] using a new triple resonance TXI HCN z-gradient CapNMR probe. Several acquisition parameters had to be considered when setting up these experiments. Only extremely low power is needed to achieve 90° magnetization rotations (<5 W). This was true for all channels independently using either 100 W ^1H or 300 W Broadband linear amplifiers. All subsequent pulse calibrations for the experiments using CapNMR probe were calculated directly without correction tables due to the high linearity of the respective amplifiers in this power range. Water suppression was either achieved using presaturation,[21,22] Watergate[23] techniques, or coherence order selective pulse sequences.[24]

The flow cell volume of the TXI HCN z-gradient microcoil NMR probe is 5 μL with an active volume of 1.5 μL. Gastight syringes (Hamilton, USA) have been used to inject between 10 and 15 μL of protein sample. Using a 10 mM sucrose sample in D_2O (4 scans, no water presaturation, 0.7 Hz line broadening), the measured S/N ratio on the anomeric proton was 31:1 (200 Hz noise window). Radio frequency homogeneity of all nuclei is comparable with regular 5 mm probes. A tremendous advantage of the microcoil probe is its ease of shimming. Because the microcoils are perpendicular to the applied B_0 field and are extremely small, their shimming is very different, yet very easily accomplished as compared to regular probes. For shimming, one only needs to adjust the low order shims, that is, x, y, $x^2 - y^2$, xy, and z.

All heteronuclear triple resonance sequences have been implemented using sensitivity enhancement.[24] The HNCA[25] and the corresponding HNCOCA[26] spectrum were comprised of 1024 × 56 × 64 complex points in the ^1H, ^{15}N, ^{13}C dimension with corresponding acquisition times of 71.3, 15.8, 7.1 ms. The relaxation delay was set to 1.3 s along with 16 scans/FID resulting in a total measurement time of ~24 h. The HNCO[25] spectrum was recorded using 1024 × 56 × 46 complex points (^1H, ^{15}N, ^{13}C), 71.3, 15.8, 12.7 ms acquisition times, respectively, 8 scans/FID, and a relaxation delay of 1.3 s. The total measurement time was ~11 h. 1024 × 46 × 64 complex points (^1H, ^{15}N, ^{13}C), with corresponding 71.3, 13.6, and 3.1 ms acquisition times, have been used to record a CBCACONH[27] spectrum. A 1 s relaxation delay and 16 scans/FID gave rise to a total measurement time of ~16 h.

Aliphatic−aromatic HCCH TOCSY spectra were recorded using a uniformly labeled $^{13}C/^{15}N$ sample in H_2O. A z-filter version of the HCCH-TOCSY[17] sequence was implemented as a 2D spectrum. T1 ^{13}C carbon evolution was completed before the TOCSY mixing step, and in the t2 time, proton evolution was developed. It is known that the FLOPSY-16[28] mixing sequence has a very broad square-shaped mixing profile, and therefore it was implemented in the HCCH TOCSY experiment. FLOPSY-16 was experimentally compared to DIPSI-2 to validate this fact. ^{13}C spin lock fields of up to 22 kHz were used to achieve the aliphatic aromatic transfer. FLOPSY-16 mixing times between 11.77 and 25.8 ms were used. 2048 × 128 complex points for ^1H and ^{13}C, respectively, were used (142.6, 3.1 ms). The relaxation delay was set to 1.5 s, and between 128 and 512 scans/FID have been used, leading to total measurement times between ~8 and 30 h. The design of the microcoil probe limits thermal heating for spin locking

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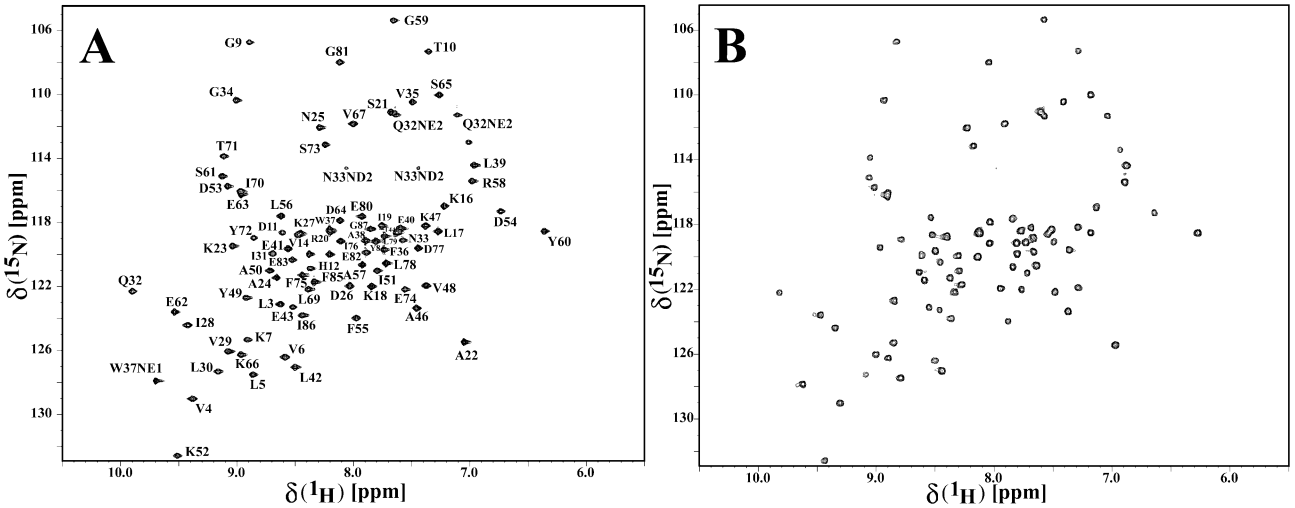

Figure 1. Comparison of the 2D [^{1}H,^{15}N] HSQC spectrum of TM0979. (A) Fully annotated 2D [^{1}H,^{15}N] HSQC spectrum recorded with a 5 mm TXI HCN z-gradient probe (Bruker, Billerica, USA) using a 2 mM sample of TM0979: 8 scans, 1k × 128 complex points, experiment time, 30 min. (B) TXI HCN z-gradient CapNMR (MRM Corp., Savoy, USA) microcoil probe using 5 μL of the 2 mM sample used in (A): 16 scans, 1k × 128 complex points, experiment time, 60 min; measurement temperature for both spectra was 313 K. Traces taken out of this spectra are shown in the Supporting Information.

and other extended pulse trains. All spectra were recorded on a Bruker DRX600 (Bruker, Billerica, USA) spectrometer.

Results and Discussion

A high-resolution single radio frequency proton-only microcoil probe has been previously described.[11] Microcoil NMR probes use solenoid coils, which have shown to have a higher intrinsic sensitivity[13] than commonly used saddle coils (Helmholtz coils) by a factor of 2−3 due to stronger coupling to the sample. The sensitivity of solenoid coils is given by eq 1:[29]

$$\frac{B_1}{i} = \frac{\mu_0 n}{d\sqrt{1+\left(\frac{h}{d}\right)^2}} \qquad (1)$$

where B_1 is the applied radio frequency field, i is the current unit, μ_0 is the permeability in a vacuum, n is the number of turns, d is the diameter of the coil, and h is the length of the coil. Therefore, as displayed in eq 1, by decreasing the diameter of the coil, an additional increase in sensitivity can be expected.[7] However, while NMR probes with saddle coils are known for high B_0 homogeneity, which is a necessary requirement for high-resolution NMR spectroscopy, solenoid coils are not. To achieve this high homogeneity also for solenoid coil-based NMR probes, the solenoid coil needs to be embedded in a perfluorinated magnetic susceptibility matching fluid. Consequently, the microcoil probes using this embedded solenoidal coil design with its characteristic small size show a tremendous proton sensitivity and inherent short pulses, which opens up a new avenue for designing pulse sequences for the assignment of proteins. Solenoidal coils can achieve much shorter pulse lengths relative to standard 5 mm Rf-coils currently used, which is of special importance for measurements at high field NMR spectrometers widely used for biomolecular NMR. For biomolecular protein NMR, the most widely used probe design is a triple resonance inverse probe with actively shielded single- or triple-axis gradients. The inner coil is tuned to ^{1}H and ^{2}H frequency with the outer coil tuned to the ^{13}C and ^{15}N frequencies. The CapNMR probe used for these experiments utilizes a quadruple tuned circuit. The coil is tuned to ^{1}H, ^{2}H, ^{13}C, and ^{15}N frequencies.

In contrast to currently used 5 mm probes for biomolecular NMR that use a vertically oriented saddle coil, the CapNMR probe is based on a flow-through design which is needed in the context of the horizontal solenoid coil design. The protein is injected into the probe by means of a gastight syringe or a small automatically driven syringe pump.

The protein BPTI was used to perform feasibility studies of acquiring protein NMR data with the CapNMR probe. It was possible to measure a 1D ^{1}H NMR spectrum with only 2.34 μg of protein in the active volume (recording time ∼20 min), corresponding to a 0.24 mM sample (Figure S2).

A well-documented set of heteronuclear triple resonance experiments provide the data for the sequential backbone resonance assignment for proteins.[19,20] This complete resonance assignment is an essential prerequisite for accurate structure determination using NMR spectroscopy. Most of these techniques are based on the 2D [^{1}H,^{15}N] heteronuclear single quantum correlation spectrum (HSQC).[30] This spectrum serves as a "fingerprint" of the protein. The well-resolved 2D [^{1}H,^{15}N] HSQC spectrum of the conserved hypothetical protein TM0979 is shown in Figure 1.

From a comparison of the 2D [^{1}H,^{15}N] HSQC spectra obtained using a 2 mM sample in both a CapNMR probe and a 5 mm triple resonance probe, it was found that the CapNMR probe had an approximately 7.5 times greater mass sensitivity (traces of these spectra can be found in the Supporting Information, Figure S3). The basis for this finding was that, while for the measurements in the 5 mm probe 110 times more sample was used (10 860 μg (550 μL): 98.7 μg (5 μL); 5430 μg (270 μL): 29.6 μg (1.5 μL)), the S/N ratio in the acquired spectra is only favoring the 5 mm probe by about 15. These results indicate that for a fixed mass in the sample volume the CapNMR probe would have a ∼7.5 times greater sensitivity, while for a fixed mass in the active volume the CapNMR sensitivity is 12.2 times greater. These mass-based sensitivities

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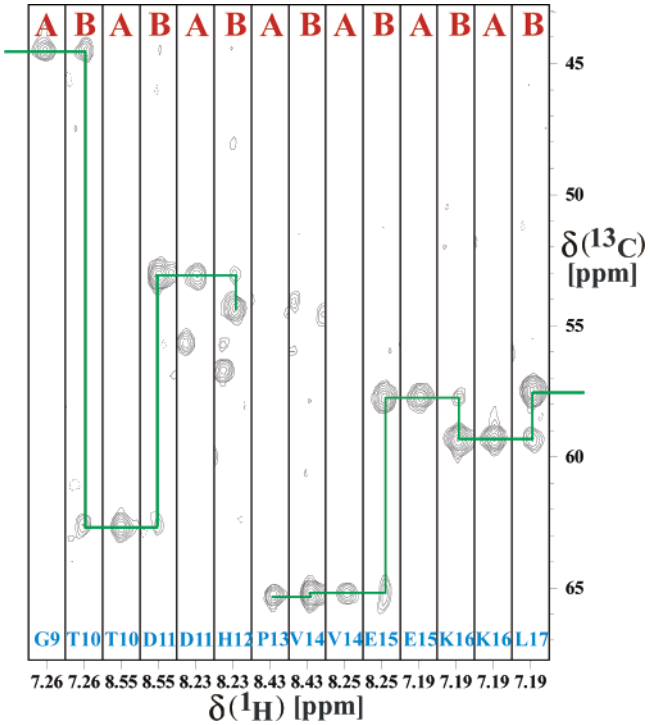

Figure 2. Fully annotated strips from amino acid glycine 9 to leucine 17: (A) HNCOCA, (B) HNCA. The sequential walk is indicated and is only interrupted by proline 13.

are much greater than the factor of 3 obtained through the use of cryotechnology. These findings are in excellent agreement with the previous described mass sensitivity increase based on the solenoid coil design and the decreased coil diameter. Shigemi microcell NMR tubes, which are widely used in biomolecular NMR spectroscopy, have the possibility to reduce the necessary sample volume of 550 μL to roughly 250−300 μL sample volume for 5 mm probes. This will reduce the amount of needed material by a factor of 2 (a table with further comparisons can be found in the Supporting Information).

The sequential backbone assignment of proteins is routinely done using either a combination of the HNCA/HNCOCA (for 5−150 kDa proteins) or HNCACB/CBCACONH (for 5−25 kDa proteins) spectra.[31,32] The assignment is based on the fact that different types of amino acids show distinctive Cα and Cβ chemical shift values that can be used to identify these amino acids in the spectra. HNCA/HNCOCA spectra have been used to test the ability to perform the sequential backbone assignment of TM0979. The measurement time of these spectra was less than 24 h. The fully annotated strips from amino acid glycine 9 to leucine 17 are shown in Figure 2. In the HNCA spectrum, all inter- and intraresidue peaks are detectable. In addition, nearly all Cα and Cβ peaks can be observed in the CBCACONH spectrum. This ensures that the combination of both spectra for the sequential backbone assignments can be pursued using the CapNMR probe. All CO correlation peaks could be identified from the HNCO spectrum. The complete backbone assignment of TM0979 has been accomplished.

The sequential assignment of the protein backbone is the essential requirement for any further interpretations of the protein. Therefore, data for the subsequent protein dynamics analysis and binding studies can be easily acquired with this probe, using only a fraction of protein than was previously required. The sequential assignment of the protein backbone is the starting point for a complete structure determination of a protein by NMR spectroscopy. The only prerequisite to perform the measurement is that the protein sample is stable at concentrations of 2 mM or more and does not aggregate.

This basic set of well-established and documented triple resonance NMR spectra for the assignment of the protein backbone is complemented by experiments used for the assignment of aliphatic side chains in proteins. In contrast to these rather quick and highly reliable procedures, the assignment of side chains of aromatic residues is time-consuming and circuitous. However, aromatic residues are one of the key factors for the stability of proteins, forming the hydrophobic core of the protein.

The most important step in the assignment process of the aromatic residues is to connect the Cβ/Hβ chemical shifts of tyrosine, phenylalanine, histidine, and tryptophan with the Hδ protons in the aromatic ring.[1] Different heteronuclear through-bond correlation spectra have been described,[33−36] although most of them lack sensitivity and ease of handling. Unlabeled protein samples, where the amide protons are completely exchanged with D_2O which reduces the chemical shift overlap in the aromatic chemical shift region, can be used to achieve the assignment of the aromatic side chains. The assignment of the proton aromatic resonances still relies on high-quality 2D [^{1}H,^{1}H] NOESY (connection between Hβ and Hδ protons), 2D [^{1}H,^{1}H] COSY, and 2D [^{1}H,^{1}H] TOCSY spectra of these completely H/D exchanged protein samples. These spectra are easy to set up, but time-consuming and, due to the rather poor dispersion, especially for phenylalanine side chains, susceptible to ambiguous chemical shift assignment. Additionally, the H/D exchange can take weeks to be completed, and the proteins under investigation need to be stable for a certain period of time.

In this paper, a method for the assignment of proton and carbon chemical shifts of aliphatic and simultaneously of aromatic side chains using a HCCH-TOCSY correlation spectroscopy is presented. The correlation between the aliphatic and aromatic carbons has been hindered until now due to the large carbon chemical shift ranges (aliphatic carbons, 0−75 ppm; aromatic carbons, 115−140 ppm). This corresponds to a total chemical shift range of ~20 000 Hz at a 14.1 T B_0 field spectrometer (~135 ppm). Traditional 5 mm NMR probe coils are not rated for the high power levels required to produce Hartman−Hahn mixing sequences needed to cover this broad chemical shift region.

An HCCH-TOCSY spectrum with a z-filter FLOPSY-16 mixing sequence using a 20 kHz spin lock field has been the basis for our measurements. The TOCSY field strength was varied between 10 and 22 kHz. This spectrum could be used for the assignment of the connection between the aliphatic Cα and Cβ atoms with the rest of the aromatic side chain and within

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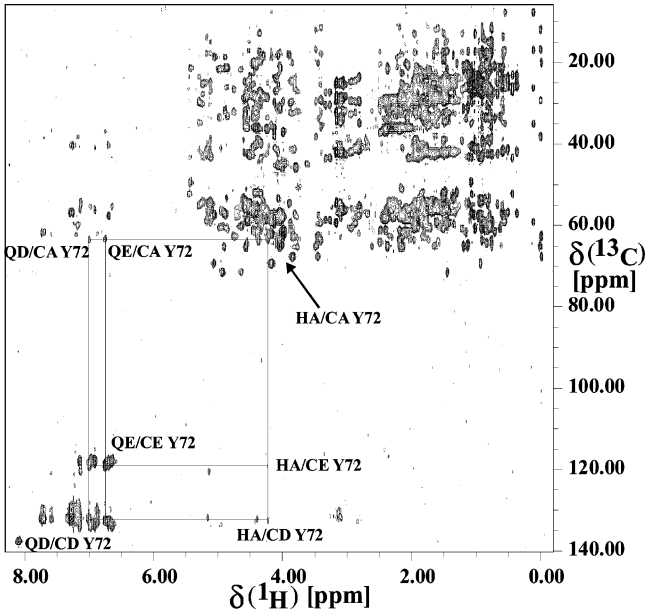

Figure 3. 2D [^{1}H,^{13}C] aliphatic−aromatic HCCH-TOCSY FLOSPY-16 spectrum recorded with a mixing time of $t_m = 11.77$ ms. The assignment of tyrosine 72 based on the Cα chemical shift is indicated in the spectrum (10 mM ^{13}C/^{15}N TM0979, 313 K, measurement time 30 h).

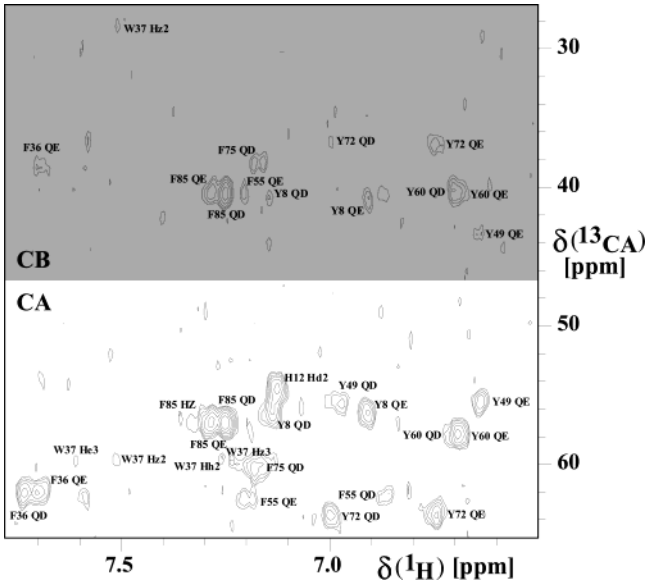

Figure 4. Enlargement of the Cα/Cβ carbon chemical shift−proton aromatic chemical shift region from the [^{1}H,^{13}C] aliphatic−aromatic HCCH-TOCSY FLOSPY-16 spectrum as shown in Figure 3. All cross-peaks are annotated, and the complete aromatic chemical shift assignment can be achieved. The Cβ cross-peak area is highlighted with a gray box.

the aromatic ring for the first time within one measurement. Interestingly, as experimentally noticed, the transfer amplitude of the TOCSY transfer favors the Cα−Cδ transfer. Therefore, the strongest cross-peaks can be seen between the Cα atoms and the Cδ(Hδ) atoms. It can be expected that the magnetization transfer during the TOCSY sequence is mostly sequential along the carbon side chain by the means of $^1J_{CCaliphatic}$ ~35 Hz, $^1J_{CCaromatic}$ ~60 Hz coupling constants, for the mixing times used for the experiments described in this paper. The $^3J_{C\alpha C\delta}$ coupling can be expected to be between 1 and 5 Hz. The corresponding mixing time was chosen between 11.77 and 29.42 ms. A detailed description of the transfer is difficult and not the aim of this work. It was seen in a previous study that for an aliphatic-only HCCH-TOCSY spectrum the best mixing time was approximately 12−14 ms with regards to sensitivity due to relaxation. Carlomagno et al.[36] showed that an average transfer time of 34 ms would be optimal for the Cβ−Caromatic transfer. We observed, as expected, a loss of sensitivity in our spectra when going to longer mixing times presumably due to relaxation. Interestingly, cross-peaks between the aliphatic carbon and aromatic protons showed a much higher sensitivity versus the cross-peaks between the aromatic carbons and aliphatic protons. The most obvious explanation for this is that almost all of the aromatic protons are degenerate, giving twice the intensity relative to the aliphatic protons. Furthermore, the aliphatic protons have an additional coupling partner, decreasing further their intensity. In addition, the aromatic carbon atoms have faster transverse relaxation than their aliphatic counterparts. Figure 3 shows a 2D aliphatic−aromatic HCCH-TOCSY spectrum.

Not surprisingly, the transfer patterns of phenylalanine and tyrosine are very similar (Figure 4). The transfer between Cα−Cδ(Hδ)/Cϵ(Hϵ) is strong, and the peaks can be easily detected and assigned. The differentiation between the Cδ/Cϵ could be done on the basis of the aromatic chemical shifts, which is especially easy in the case of tyrosine moiety. For phenylalanine, only one transfer to Cζ could be detected for F85. Of particular interest is that, for the tyrosine moiety, a rather strongCβ−Cδ(Hδ)/Cϵ(Hϵ) cross-peak could be detected. The comparative intensities between different mixing times do not change to a great extent. For some residues, the Cβ−Cδ(Hδ)/Cϵ(Hϵ) gets weaker at longer mixing times, presumably due to ongoing T_2 relaxation.

For histidine, a strong Cα−Cδ2(Hδ2) cross-peak could be detected at a mixing time of 11.77 ms. The Cβ−Cδ2(Hδ2) could only be detected at a longer mixing time of 29.42 ms. Nevertheless, this Cβ−Cδ2(Hδ2) cross-peak has a high sensitivity at this long mixing time. This behavior was only seen for the histidine spin system. Here, the magnetization cannot flow to the Cδ2 carbon and back, because the nitrogen atoms Nδ1 and Nϵ2 will block the transfer. For the tryptophan residue, stronger transfer could only be detected for the Cα−Cδ1(Hδ1) and Cβ−Cζ2(Hζ2) cross-peaks. All other cross-peaks were very weak. All of the aromatic side-chain protons could be assigned on the basis of their different chemical shifts. In most cases, for the differentiation of cross and diagonal peaks in the carbon aromatic region, an additional constant time 2D [^{1}H,^{13}C$_{arom}$] HSQC spectrum was used.

In general, the presented spectrum allows for all aromatic side chains to be easily assigned. This will allow for the complete chemical shifts of the aromatic carbon and hydrogen nuclei to be assigned simultaneously. Moreover, the connection between the Cβ atom and the aromatic ring of the amino acids and the assignment can be done at once. If an overlap of peaks makes the assignment difficult, a 3D version using a second proton evolution time could be easily implemented. This increased dimensionality was not necessary for the assignment of the TM0979 protein.

Conclusions

The TXI HCN z-gradient CapNMR probe presented in this paper opens two important possibilities for high-resolution NMR

spectroscopy. First, the probe allows one to acquire NMR data on microgram amounts of protein, which has not been previously possible without using NMR tube inserts or plugs. Second, a new possibility for the assignment of aromatic side-chain chemical shifts in proteins is presented. The Rf characteristics of the CapNMR probe have now made it possible to record a single HCCH TOCSY spectrum across the full aliphatic and aromatic side-chain carbon range.

The intrinsic sensitivity of the microcoil probe is comparable to that of the 5 mm cryoprobes if concentrated samples are used. As shown in this study, a 10 mM sample of a ~10 kDa protein allowed the measurements of heteronuclear triple resonance spectra in a very short period of time. It is predictable that for samples with concentrations lower than 2 mM this would be rather difficult and perhaps impractical due to extremely long measurement times. Nevertheless, simple 1D and 2D spectra like 2D [^{1}H,^{15}N] HSQC's can be recorded on very dilute samples. We have shown that on samples containing as low as 10 μg of protein, we could easily acquire HSQC's. This should allow for a rapid and very detailed biophysical description of small amounts of unlabeled and labeled proteins that was, until now, impossible.

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Supporting Information Available: Additional figures showing the complete sequential backbone assignment of TM0979, representative traces from the 2D [^{1}H,^{15}N] HSQCs recorded with a Bruker TXI HCN 5 mm probe and the microcoil TXI HCN probe, and a 1D ^{1}H spectrum recoded with 2.34 μg of BPTI in the active volume. This material is available free of charge via the Internet at http://pubs.acs.org.

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