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Using cryoprobes to decrease acquisition times of triple-resonance experiments used for protein resonance assignments

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Abstract. In most structural biology NMR laboratories, instrument time is a limiting factor in the number of structural projects a laboratory is able to support. In the post-genomic era we can expect the number of structural targets to markedly increase. Here we address to what degree recently introduced cryoprobes, which are 3–4 times as sensitive as conventional probes, can alleviate this problem. To evaluate this approach, a set of triple-resonance experiments for protein assignments were acquired with a cryoprobe. We show that, with the cryoprobe, high quality triple-resonance data can be obtained within as 4 hours/experiment. These results show that a full set of data for protein assignments can now be practically collected in 1–2 days.

Keywords: NMR spectroscopy, cryoprobes, triple-resonance, proteins, high-throughput, reduced phase cycle

1. Introduction

In recent years, significant advances in NMR probe technology have been achieved. One of the most exciting has been the practical commercialization of cryoprobes. Cryoprobes offer up to a 4-fold increase in sensitivity compared to conventional probes [11,12]. Potentially, the improved sensitivity translates into a 4-fold reduction in sample concentration or, conversely, a 16-fold reduction in experimental times, at sample concentrations used in conventional probes. Clearly, this development will greatly impact many areas of NMR, including natural product analysis, high throughput screening, and SAR by NMR [10]. Structural biology laboratories using NMR will also benefit from the introduction of cryoprobes. First, by taking advantage of being able to work at lower concentrations, the cost of preparing uniformly ^{15}N/^{13}C/^{2}H-labeled samples will be markedly reduced. Furthermore, the reduction in concentrations will make samples that exhibit concentration dependent aggregation more accessible. Secondly, it should also be possible take advantage of the potential savings in experimental times to dramatically decrease the time required for structural analysis of proteins by NMR.

The contemporary method for determining protein structure in solution uses the sequential assignment of backbone and side-chain resonances obtained from a basic suite of scalar-coupled triple-resonance experiments, i.e. HNCACB, CBCA(CO)NH, etc. [2]. Once the assignments are obtained analysis of nOe and coupling constant data generates an initial set of constraints that are used to begin calculating the structure of the protein (Wuthrich, 1986). Currently, for small to medium sized proteins, triple-resonance experiments can be acquired in 2–3 days each. As a result it requires almost 2 weeks of instrument

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time to collect the full suite of data required for complete resonance assignments. On the other hand, in favorable cases an experienced investigator can analyze each data set in a few hours and complete the backbone resonance assignments in the same amount of time. Therefore, NMR instrument time becomes the limiting resource. A common problem for structural biology laboratories is not a lack of structural targets, but a lack of available NMR instrument time. To overcome this in the past investigators either purchased additional NMR instruments or arranged for time on instruments outside their laboratories. For various reasons either one or both of these solutions are not always possible. We propose that by working with a cryoprobe, at sample concentrations used for conventional probes triple-resonance experiments can be reduced to a minimal phase cycle such that only 1 or 2 scans/increment would be required. This paper addresses the technical issue of whether this is currently practical.

2. Results and discussion

To test this approach we acquired several triple resonance experiments used for protein assignments on a Bruker DMX500 equipped with a cryoprobe (Bruker Instruments, Billerica, MA) utilizing the Cε2 domain from human IgE, which is currently under structural analysis in our laboratory [6]. The experiments that were run include HNCA, HNCACB, CBCA(CO)NH, HBHA(CBCACO)NH, HN(CA)CO, and HCCH-TOCSY. The sample conditions were, 280 μl of 1.9 mM protein (MW 11.5 kDa), 100 mM sodium phosphate, pH 6.0, 20°C and 10% D_2O in a Shigemi NMR-tube (Shigemi, Allison Park, PA). The pulse sequences used here were gradient-selected sensitivity-enhanced versions [5,9]. An advantage of using pulsed-field gradients (PFG) for coherence selection is that, provided the signal-to-noise is sufficiently high, it is possible to collect multi-dimensional data with only 1 scan/increment [1,3,5]. To ensure this condition was met a high protein concentration was chosen for this initial study. However, the quality of the data obtained indicates we are not limited to these high concentrations and that a protein concentration of 1 mM would have been sufficient. It should be noted that we employed pulse sequences directly from the standard Bruker pulse sequence library without any further modifications. When implementing these experiments, the number of scans per increment was simply reduced to the desired value without the addition of any PFGs around the pulses that were no longer phase cycled. Also, all of the data discussed here were acquired during a single 48-hour period at the Bruker facility in Billerica, MA.

In theory it is possible to make sequential backbone assignments from the HNCACB and CBCA(CO)-NH experiments, which were the first experiments to be tested. The full 3D-data matrices for these two experiments were acquired using 2 scans/increment, the total acquisition time for each experiment was 4 hours (further experimental details are provided in the figure legends). Representative data from the HNCACB and CBCA(CO)NH experiments collected using the cryoprobe are shown in Figs 1 and 2. The Fig. 1 shows sequential amide proton strips for Asp47–Asp52 extracted from the full 3-dimensional data matrices. The resonance assignments for this protein were previously determined exclusively from data collected using a conventional probe. Clearly the data acquired using the cryoprobe are of high quality and can be used for resonance assignments. A comparison of the initial conventional-probe data (data not shown) with this cryoprobe data confirms, as expected, that the information content of these data sets are the same. The 1D-traces through Met51 (Fig. 2) further illustrates the signal-to-noise achieved for these experiments. Although the sample concentration was 1.9 mM, from these traces it is obvious that these experiments could have been run with a protein concentration of 1 mM.

By using PFGs for coherence selection and artifact suppression it is theoretically possible to acquire data in as little as 1 scan/increment [1,3,5]. However, several potential problems need to be considered

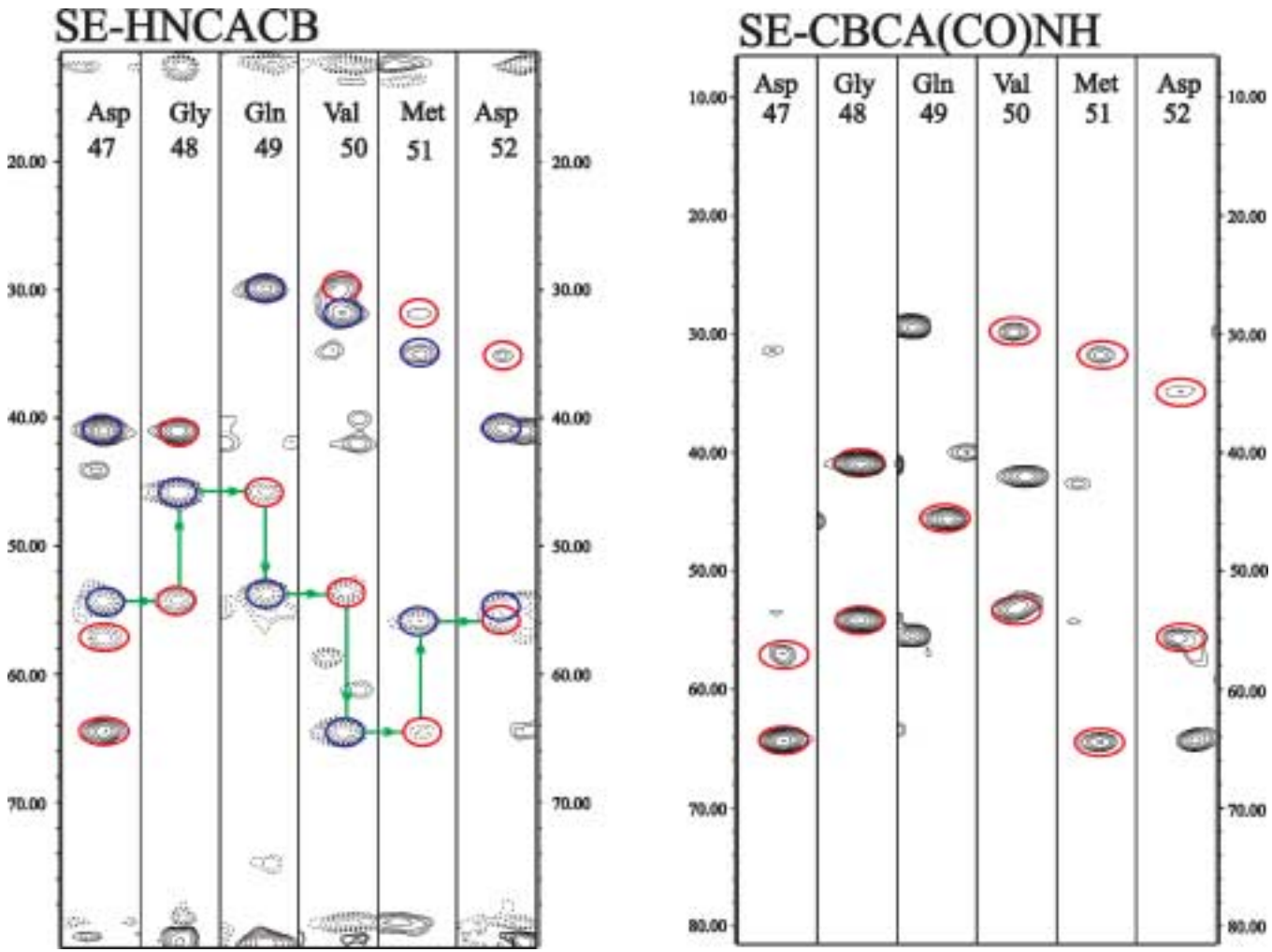

Fig. 1. HNCACB and CBCA(CO)NH strip plots showing sequential assignments from D^{47} to D^{52}. The HNCACB data was acquired with the following parameters; 128* (t1) × 50* (t2) × 1024* (t3) complex points, 8802.8 Hz (t1) × 2027.4 Hz (t2) × 7002.8 Hz (t3) sweep widths, and zero-filled to a final matrix size of 256 (t1) × 128 (t2) × 1024 (t3). The CBCA(CO)NH data was acquired with the following parameters; 128* (t1) × 50* (t2) × 1024* (t3) complex points, 94343.0 Hz (t1) × 2027.4 Hz (t2) × 8503.4 Hz (t3) sweep widths, and zero-filled to a final matrix size of 256 (t1) × 128 (t2) × 1024 (t3). Both spectra were acquired in 4 hours using 2 scans/increment on a Bruker DMX500 equipped with a cryoprobe. For the HNCACB the Cα peaks are plotted as negative contours by dashed lines. Intra- and inter-residue correlations are indicated be the heavy-dashed lines and solid lines, respectively. Sequential assignments are indicated by the arrows.

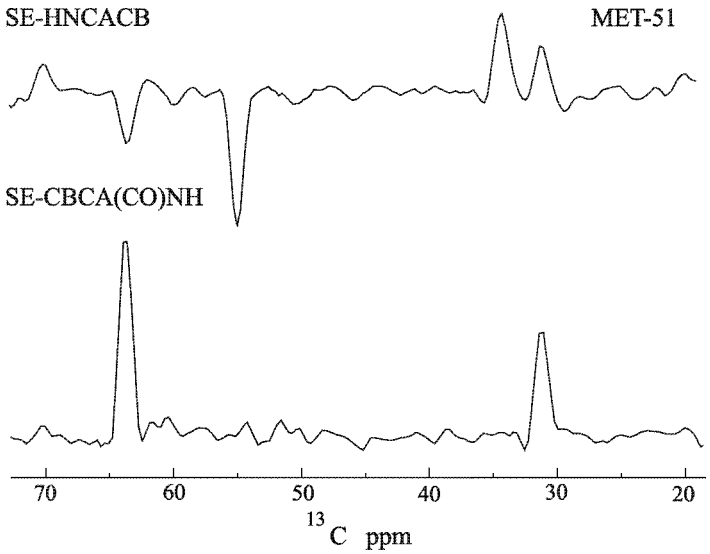

Fig. 2. 1D-traces extracted from the HNCACB (A) and CBCA(CO)NH (B) data shown in Fig. 1 at the amide-^{1}H resonance of Met51.

when implementing triple-resonance experiments in this manner. One has already been addressed; that at protein concentration of 1 mM the signal is strong enough to be acquired in only 1 or 2 scans. Additionally one also needs to consider the issues of solvent and axial peak suppression. The larger number of scans/increment used when running triple-resonance experiments on conventional probes makes it a trivial matter to include additional phase cycling to improve solvent and axial peak suppression. However, it has been shown that these phase cycles for artifact suppression can be removed by a judicious use of PFGs [1].

Our first concern about the implementation of these pulse sequences, as described above, was for the severity of any axial peaks, as are observed in the HNCACB (Fig. 1) at 2 scans/increment. However, the intensity of the observed axial peaks are not prohibitive and by increasing the sweep width in States-TPPI mode (Marion et al., 1989) they are moved far enough away from the resonances of interest as to not interfere with data analysis. It can be noted that axial peaks were only observed for the HNCACB experiment and not in any of the other experiments tested (Fig. 3). The reason for this is undoubtedly due to the reduction of the 16-step phase cycle in the experiment from the Bruker pulse sequence library, to 2-steps without the addition of any PFGs around the pulses no longer phase cycled [1]. This is supported by the lack of artifacts observed in the CBCA(CO)NH data, in which phase cycle of the experiment from Bruker pulse sequence library had already been reduced by additional PFGs.

We were also concerned about the quality of the water suppression for these experiments. We found that at 1 scan/increment water suppression was unacceptably poor, such that it was impossible to observe amide-^{1}H signals under these conditions (data not shown). When the scans/increment were increased to 2 (2-step phase cycle), there was a marked improvement the water suppression, and the expected amide-^{1}H signals were observed. This can be attributed to the simple cancellation of undesirable signals brought about by the phase cycle. Part of the difficulty associated with the water suppression can be attributed to restricted homogeneity of the samples. The water resonance was broad with a half-height width of 50–60 Hz, and rather non-Lorentzian lineshape. Both standard and Shigemi NMR tubes were tried; the results were that we observed similar, but slightly better, water suppression with the Shigemi tube. It is

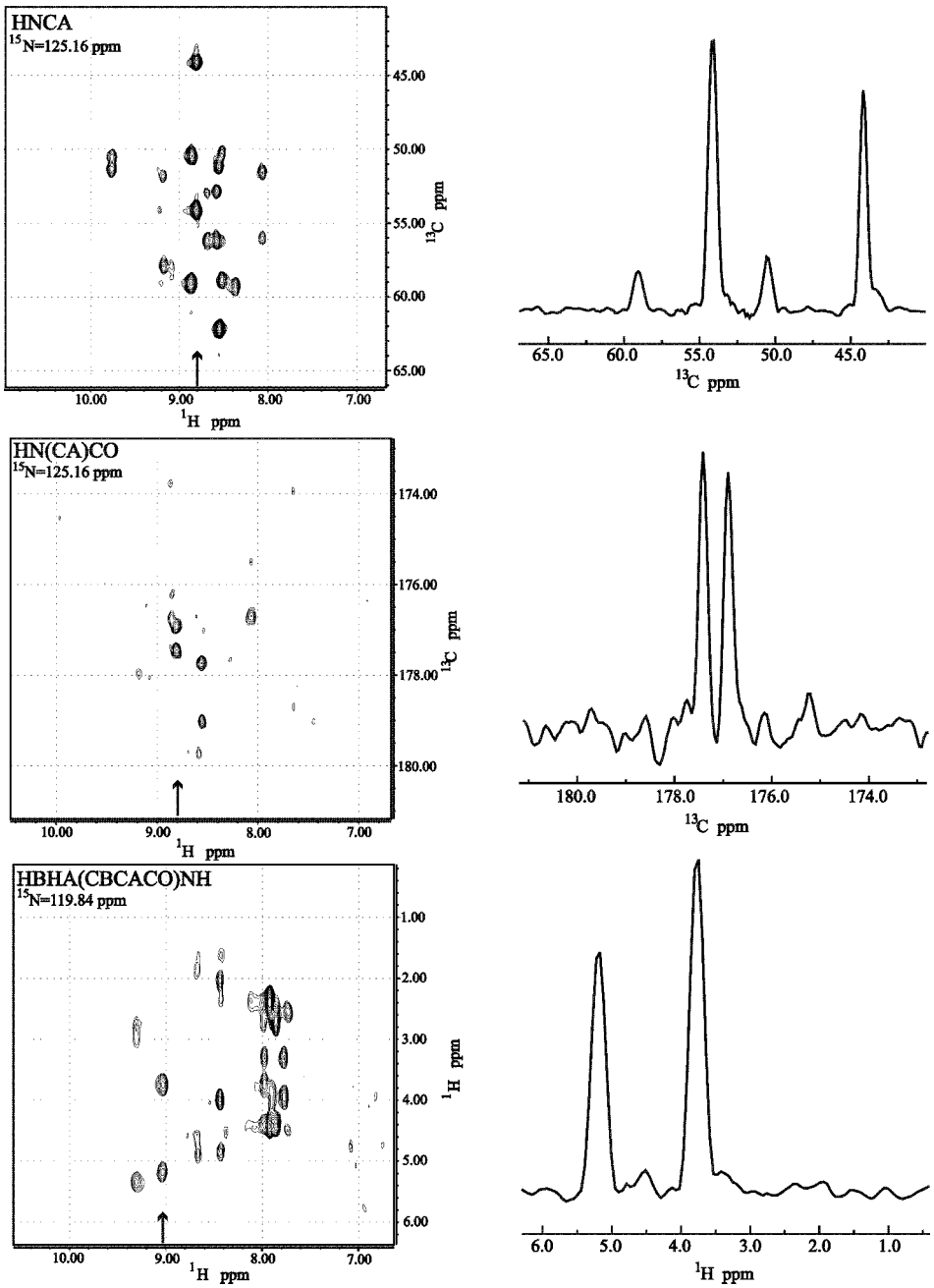

Fig. 3. Selected regions of 2D-planes from the HNCA, HN(CA)CO, and HBHA(CBCACO)NH data matrices. The HNCA data was acquired with the following parameters; 128* (t1) × 50* (t2) × 1024* (t3) complex points, 4032.3 Hz (t1) × 2027.4 Hz (t2) × 8503.4 Hz (t3) sweep widths, and zero-filled to a final matrix size of 256 (t1) × 128 (t2) × 1024 (t3). The HN(CA)CO data was acquired with the following parameters; 128* (t1) × 50* (t2) × 1024* (t3) complex points, 1509.4 Hz (t1) × 2027.4 Hz (t2) × 7002.8 Hz (t3) sweep widths, and zero-filled to a final matrix size of 256 (t1) × 128 (t2) × 1024 (t3). The HBHA(CBCACO)NH data was acquired with the following parameters; 128* (t1) × 50* (t2) × 1024* (t3) complex points, 7002.8 Hz (t1) × 2027.4 Hz (t2) × 7002.8 Hz (t3) sweep widths, and zero-filled to a final matrix size of 256 (t1) × 128 (t2) × 1024 (t3). The 1D-traces (B,D,F) were extracted at the amide-^{1}H chemical shift indicated by the arrows in each of the corresponding 2D-panels (A,C,E), respectively.

Table 1
Comparison of experimental times

Experiment[1,2]	Conventional		Cryoprobe	
	# scans/FID	Exp. time	# scans/FID[4]	Exp. time
HNCA	16	31 hrs	2	4 hrs
CBCA(CO)NH	16	31 hrs	2	4 hrs
HNCACB	32	62.5 hrs	2	4 hrs
HBHA(CBCACO)NH	32	62.5 hrs	2	4 hrs
HCCH-TOCSY[3]	16	75 hrs	2	9.5 hrs
	Totals	262 hrs		25.5 hrs
		∼11 days		∼1 day

[1]A relaxation delay of 1.1 s was used for all calculations.
[2]Experimental times were calculated using 64 complex × 25 complex points in the indirect dimensions.
[3]Experimental time was calculated using 92 complex × 40 complex points in the indirect dimensions.
[4]The 11.5 kDa protein sample used for data collection on the cryoprobe was concentrated to 1.9 mM in 100 mM sodium phosphate, pH 6.0 at 20°C.

expected that the water suppression in these experiments would be improved by implementing flip-back techniques to maintain the water resonance along the z-axis throughout the pulse sequence [4].

Figure 3 shows data extracted from the full 3D-data sets from the other experiments tested, including the HNCA, HBHA(CBCACO)NH, and HN(CA)CO sequences. As with the previous experiments, these were acquired with total experimental times of 4 hours each. Traces through these data further illustrate that it is possible to obtain very high quality data under these experimental conditions when employing a cryoprobe. These data were chosen as representative examples of these experiments and show that reduction in sample concentration to 1 mM would not result in any loss of the informational content of these experiments. The one exception to this might be the HN(CA)CO as implemented here. The 1D-trace from this experiment reveals that the signal-to-noise ratio for this experiment is much less than that of the other experiments tested, but when we examined the full data matrix of each of these three experiments we again found that these data were usable for the assignment of this protein. In fact the HN(CA)CO had not been previously acquired for this protein, and it was used in conjunction with an HNCO experiment acquired on a conventional probe, to confirm the previously determined sequential assignments obtained from HNCACB and CBCA(CO)NH experiments.

These results demonstrate that with the increased sensitivity of the cryoprobe, triple-resonance experiments can be acquired in a fraction of the current experimental times. The cumulative effect is that all experiments discussed here can be run in 25 hours (Table 1). On a conventional probe the same 6 experiments would have required 262 hours (11 days) of instrument time. This is an 11-fold saving in time, which is close to the expected reduction in experimental times of 16-fold. The explanation for this deviation is found in the requirement of needing 2 scans/increment to achieve acceptable solvent suppression. Therefore, for a very sensitive experiment, such as the CBCA(CO)NH, there is only an 8-fold reduction in experimental time because this experiment can be run with 16 scans/increment on a conventional probe. Whereas for other experiments that were acquired with 32 scans/increment on a conventional probe the full 16-fold reduction in experimental times is achieved. However, it should be possible that the pulse sequences can be optimized for artifact and solvent suppression so that only 1 scan/increment would be required.

3. Conclusion

It is clear that commercial cryoprobe technology is a major improvement in NMR probe sensitivity. We have shown that with a cryoprobe current experimental times for triple-resonance experiments can indeed be reduced up to 16-fold. The dramatic reduction in experimental times will greatly alter how NMR structural biologists think about and develop projects. Now, with a cryoprobe, an HNCA, HN(CO)CA, HNCACB, CBCA(CO)NH, HNCO, HN(CA)CO, CC(CO)NH, and HCC(CO)NH experiments can all be acquired in less than 2 days. Over the course of a weekend all the experimental data needed to make complete, non-aromatic, backbone and side-chain assignments can be collected. Clearly this is a fundamental shift in the rate determining step of structure determination by NMR from data collection to data analysis. In the past, analysis of one of these data sets could be accomplished, even in fully manual-mode, in just a few hours, long before the next data set in the series was collected. With this increase in data collection rates, robust and reliable methods for automated resonance assignments become an absolute requirement for NMR to have an impact in areas such as structural genomics [8].

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