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Historical Perspective

Triple resonance three-dimensional protein NMR: Before it became a black box

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ABSTRACT

Three-dimensional triple resonance experiments have become an integral part of virtually every solution NMR study of proteins. The approach relies on uniform isotopic enrichment of proteins with ^{13}C and ^{15}N, and establishes the scalar connectivity pathway between nuclei through the large $^{1}J_{NH}$, $^{1}J_{CH'}$ $^{1}J_{CC}$, and $^{1}J_{CN}$ couplings. The magnetization transfer process takes place through multiple, efficient one-bond magnetization transfer steps, rather than a single step through the smaller and variable $^{3}J_{HH}$ couplings. The relatively large size and good uniformity of the one-bond couplings allowed the design of efficient magnetization transfer schemes that are effectively uniform across a given protein, nearly independent of conformation. Although conceptually straightforward, practical implementation of three-dimensional triple resonance experiments on proteins originally posed serious challenges. This account provides a personal perspective on some of the historical background to this work, the problems encountered as well as their solutions, and their evolution into today's standard arsenal of experiments.

Interview with the author(s).

A video interview with the author(s) associated with this Historical Perspective and the original article can be found in the online version, at doi:10.1016/j.jmr.2011.08.003.

Today, triple resonance NMR spectroscopy of isotopically enriched proteins has become the standard approach for making resonance assignments, prerequisite to studying protein structure and dynamics. Our early work in this area played an important role in the development of this technology, with many of the practical details described in a paper that appeared in the Journal of Magnetic resonance, more than 20 years ago [1]. Below, I present some of the context of this work and a personal perspective on how it emerged from being at the right place, at the right time, surrounded by the right colleagues.

By the late 1980s, the potential power of NMR spectroscopy to study details of the atomic structure and dynamic properties of proteins had become abundantly clear. A systematic procedure for making residue-specific ^{1}H assignments had been described by the Wuthrich group [2], and together with several variations on this strategy [3–6] it was proven to be an effective method for analysis of proteins as large as about 100 residues [7–9]. However, the high degree of resonance overlap and degeneracy in such homonuclear 2D $^{1}H—^{1}H$ spectra made determination of unique and self-consistent resonance assignments a labor-intensive, time-consuming task, somewhat analogous to solving a highly complex jig-saw puzzle. Much of the analysis relied on overlaying very large, frequently 100×100 cm or even bigger paper plots of NOESY, COSY, relayed COSY and/or TOCSY/HOHAHA spectra on a lightbox, and connecting matching peaks with penciled lines – a procedure that often involved many iterations and extensive use of erasers before a consistent solution was reached. Although I was duly impressed by the terrific skills that many of these homonuclear 2D NMR practitioners had acquired at solving assignment problems, after a few failed attempts I realized quickly that this kind of work was not my calling.

Instead, my NMR efforts mostly focused on development and application of heteronuclear $^{1}H—^{13}C$ and $^{1}H—^{15}N$ experiments for the study of smaller molecules, including peptides, natural products and small oligonucleotides, mostly at natural abundance. However, inspired by elegant residue-selective ^{15}N labeling experiments carried out by Redfield, Poulter, and a number of others [10–12], I had become intrigued by the application of heteronuclear NMR experiments to the study of large biomolecules. Through a fortunate coincidence, while conducting post-doctoral research in Gary Maciel's group at the "Mile-High Proton Enhanced Nuclear Induction Spectroscopy" Center in Colorado focusing on esoteric experiments such as magic angle flipping [13] and hopping [14], I was drawn into solution NMR studies on selectively labeled tRNA, carried out next door in the Center by Griffey and Hawkins. This experience clearly showed me that, except for inherently lower sensitivity and prob-

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Fig. 1. Dominique Marion (left) and Lewis Kay, carrying eight 5-Mb disks containing the time domain data of a single 3D ^{15}N NOESY-HMQC experiment from the Nicolet 500 MHz spectrometer to a SUN work station, for processing with their in-house software.

lems with suppression of the humongous water signal, many of the small molecule experiments, pioneered in the groups of Ernst et al. [15] and my graduate mentor Freeman [16], would be applicable to biological macromolecules. Of course, isotopic enrichment, better probes, and higher fields could largely solve the sensitivity problem, but handling the intense water signal remained problematic before the introduction of commercial equipment for applying pulsed field gradients in the mid-1990s. Initially, I and many others frowned upon the isotopic enrichment concept, as it was considered "cheating", only applicable to proteins that had been cloned and could be overexpressed in *E. coli*. However, after my move to the National Institutes of Health, where I shared a laboratory with Dennis Torchia, he quickly convinced me that cloning and overexpression was likely to become the method of choice for generating pure and homogeneous samples suitable for NMR studies of a wide range of interesting systems. While Torchia's work focused mostly on solid-state NMR of the enzyme staphylococcal nuclease (SNase), he and his staff prepared their own isotopically enriched samples for these studies. This protein, of about 17 kD in mass, was clearly too large for conventional homonuclear NMR, in particular considering that 11.7T was the strongest available magnet to us at that time. However, collaborating with Dennis, we were able to establish validity of the idea that many of the "small molecule NMR experiments" indeed would be applicable to isotopically enriched proteins too. Most of these initial applications focused on isotope editing and filtering, allowing the generation of simplified NMR spectra involving only the labeled residues.

Realizing how efficient and sensitive 2D heteronuclear ^{1}H—^{15}N and ^{1}H—^{13}C correlation experiments were for enriched proteins, it was clear that the tremendous spectral overlap encountered in 2D NOESY spectra could efficiently be lifted by recording experiments in 3D rather than 2D. This concept had just been introduced and demonstrated in homonuclear ^{1}H NMR [17,18]. By good fortune, two top-notch post-doctoral associates then working in my group, Dominique Marion and Lewis Kay, took an active interest in developing this technology "in house". Recording of large 3D spectra, filling as many as 12 hard exchangeable disks with raw time domain data (Fig. 1), proved straightforward with our older Nicolet 500 MHz instrument. Development of software for carrying out the processing, viewing, and analysis of these data proved more difficult, but nothing that Marion and Kay could not handle [19]. Generation of ^{15}N-separated 3D NOESY and HOHAHA spectra rapidly became the method of choice for proteins that could be isotopically enriched. Marius Clore and Angela Gronenborn, who joined NIH in the late 1980s, aided by their highly skillful associate Paul Driscoll, were able to analyze a pair of such 3D spectra for interleukin-1β, a protein nearly twice as large as previously assigned proteins at that time [20]. Powerful as it was, the limitations of the approach were quite clear: with the increase in protein size, the inherent ^{1}H line width becomes larger than the ^{1}H—^{1}H J couplings, making the $^{3}J_{HH}$ based experiments less effective, in particular in α-helical regions of proteins where $^{3}J_{HNH\alpha}$ values are small ($\leqslant$5 Hz).

While Kay and Marion were developing and testing 3D ^{15}N-separated NOESY and HOHAHA experiments, Mitsu Ikura had joined the group to continue his study of calmodulin, a highly α-helical protein with rather poor resonance dispersion. His PhD work had focused on homonuclear 2D NMR studies of the isolated N- and C-terminal domains of this protein [21,22], with work on the intact protein mostly beyond reach of that technology. Even with the new 3D ^{15}N separated experiments, calmodulin assignments proved a very tough nut to crack, but inspired by the work of Kainosho and Tsuji [23], he decided to explore the idea of residue-selective ^{15}N labeling and ^{13}C′ labeling, allowing unique identification of ^{1}H—^{15}N HSQC cross peaks by their $^{1}J_{CN}$ splitting if the preceding residue was ^{13}C′ enriched [24]. It quickly became clear that making large numbers of selectively labeled samples was a painful and expensive proposition, and that use of uniform labeling with the less expensive U—{^{13}C} glucose and $^{15}NH_4Cl$ precursors could provide a more practical approach. However, a correlation of an ^{1}H—^{15}N amide group with the ^{13}C′ of its preceding residue, requiring development of the HNCO pulse sequence, in itself would not be very useful as it would not provide sequential assignment information. On the other hand, we argued that if it were possible to correlate $^{1}H^{\alpha}$—$^{13}C^{\alpha}$ pairs with their intraresidue ^{13}C′ resonance by means of an HCACO type experiment, this would allow us to link $^{1}H^{\alpha}$—$^{13}C^{\alpha}$ pairs of residue *i* with the amide of resonance *i* + 1, based on their identical ^{13}C′ frequency. The effectiveness of ^{13}C—^{13}C magnetization transfer in uniformly ^{13}C enriched proteins had just been demonstrated by the Markley group [25], and it appeared pretty obvious to us that this could be an effective strategy for assigning larger proteins.

The only snag in realizing our ideas was the lack of suitable spectrometer hardware. Our experiments would need to rely on ^{1}H detection to gain optimal sensitivity, and the type of dual ^{13}C/^{15}N probe with ^{1}H decoupling capabilities available at that time was not going to give us the sensitivity and ^{1}H line shape needed to carry out experiments on dilute proteins in 90% H_2O. Fortunately for us, Torchia had already anticipated this need and had asked Bruker to develop a ^{1}H-optimized triple resonance probehead, a request that required all of our combined persuasive power with Bruker to convince them to develop such a "useless" device.

With Marion's and Kay's software in place to process and analyze 3D spectra, the last missing link was a spectrometer that could generate the requisite pulse sequence. Neither the Nicolet spectrometer nor Torchia's new AM500 machine, was equipped with a programmable, phase-controlled third frequency channel, necessary to generate the requisite ^{13}C and ^{15}N pulses, while detecting ^{1}H. Moreover, even though Torchia's new AM500 offered superior ^{1}H sensitivity and line shape, it was ill-suited for 3D NMR as after collection of each FID, the spectrometer would require an 8s overhead time to write this data to the disk and to restart the experiment for collection of the next FID. This lack of suitable hardware would have posed an insurmountable stumbling block had it not been for the presence of electronics engineer Rolf Tschudin, lured away from Varian some 15 years earlier by Ted Becker when expanding the NMR research program at NIH. Designing and building a "third channel", including a phase shifter, composite pulse decoupler, and their control by the Bruker pulse programming unit, proved to be a cinch for Tschudin, who largely relied on recycled synthesizers and amplifiers of Torchia's solid-state spectrometers and a patchwork of switches, high power directional couplers, selective filters, and a clever computer-controllable phase shifter (Fig. 2).

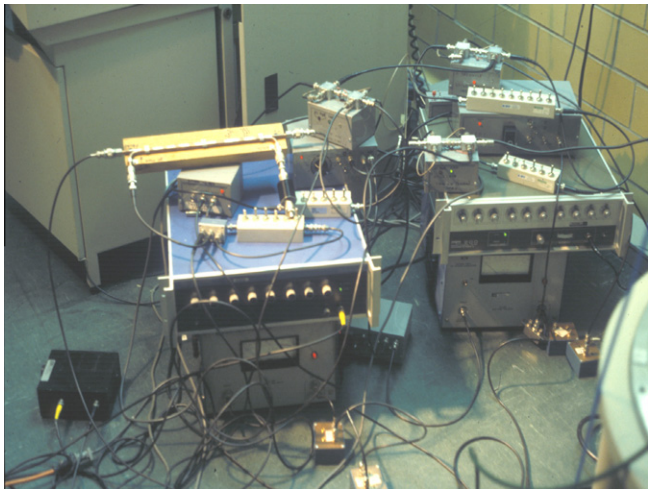

Fig. 2. Hardware interfaced to Dennis Torchia's Bruker AM500 console for generating a computer-controlled third and fourth channel, needed for execution of the early generation of triple resonance NMR experiments.

After a couple of "false starts", the big day arrived and Kay managed to generate two "superb" sets of HNCO and HCACO spectra, and proudly handed over the printouts of his efforts to Ikura. However, to our mutual surprise and frustration, even a week later Ikura had failed to "deliver the goods", and he claimed the spectra were insufficient for making unambiguous sequential assignments. Calmodulin proved a particularly challenging case, because it contains four quite homologous repeats in its amino acid sequence, and the key problem proved to be that the $^{13}C'$ frequency was insufficiently unique to unambiguously link sequential $^{1}H^{\alpha}$—$^{13}C^{\alpha}$ and ^{1}H—^{15}N pairs. Typically as many as half a dozen $^{13}C'$ having chemical shifts could not be distinguished at the ^{13}C resolution available from the 3D spectra. At this point we faced the question: Shall we go ahead and publish what we have, or proceed and develop additional experiments to provide a second, independent link to connect adjacent residues? We decided for the latter option, using the ^{15}N nucleus as the second link while generating a "relay" experiment, termed HCA(CO)N, that would extend the HCACO experiment by transferring magnetization to its sequential ^{15}N nucleus via the $^{1}J_{NC'}$ coupling, and after ^{15}N evolution, back to H^{α} for ^{1}H detection. Together with the ^{15}N frequency from the HNCO spectrum, this indeed resolved most of Ikura's dilemmas. As an additional stroke of good fortune, we found that it was also quite easy to correlate the amide ^{1}H—^{15}N pair with $^{13}C^{\alpha}$ in the HNCA experiment. Although initially, this experiment only yielded the intraresidue $^{13}C^{\alpha}$, correlated to its adjacent ^{15}N via the $^{1}J_{NC\alpha}$ coupling, we later realized that by shortening the de- and rephasing $J_{NC\alpha}$ delay, correlations to both the intraresidue and the preceding $^{13}C^{\alpha}$ could be observed, providing yet another mechanism to link adjacent amide groups.

Finding good names for our experiments presented a final challenge, before we could submit our work for publication. Trained by Ray Freeman, I knew that appropriate naming of an experiment – preferably as some sort of self-deprecating acronym – would be key to the eventual success of the method. Although we were able to float a couple of creative and potentially suitable names for what we eventually named HCACO and HNCO, we realized this would become unmanageable with the further development of closely related sequences. We therefore capitulated, and simply decided on the not very creative but perhaps more functional naming of the experiments by the atom names being correlated.

Our first attempt to publish the new assignment strategy, highlighted for the unequivocally challenging calmodulin case, proved more difficult than anticipated. The paper was initially rejected from Biochemistry, and as one of the referees put it, the "contention that this approach will replace the ones currently used seems somewhat premature and even a bit arrogant in light of the significant cost involved." Fortunately for us, the cost of ^{13}C has dramatically come down over the past 20 years, and most protein assignments are now carried out by triple resonance NMR.

While protesting the decision with Biochemistry, and eventually succeeding [26], we realized that if we wanted this approach to take root, a detailed description of the pulse sequences, the hardware, and the practical aspects involved with carrying out such experiments was called for. The Journal of Magnetic Resonance was the logical place for publishing this account, and through the kind care of Wallace Brey, the paper was rapidly accepted for publication and appeared in print soon thereafter [1].

Looking back at the way the original pulse sequences were implemented, it is quite clear that the design of these experiments was guided by limitations of the available hardware. As I had learned the hard way during my original adventures with indirect detection probes, radiofrequency homogeneity on these early generation probes was abysmal, and in particular the application of 180° pulses generated a considerable loss in sensitivity. Moreover, in the absence of sufficient scans per FID to carry out phase cycling to remove artifacts caused by imperfections of these pulses, and lacking pulsed field gradients, these original sequences were optimized to contain a minimal number of 180° pulses. A somewhat novel feature of these experiments was the "out-and-back" nature of their magnetization transfer pathway, where magnetization that initiated on H^{N} or H^{α} would be transferred in multiple steps to probe evolution of a nucleus up to four bonds away, before back transfer to the initial nucleus, which was observed during detection of the FID. This approach turns out to be quite effective, frequently requiring fewer pulses than unidirectional magnetization transfer, in particular when using heteronuclear multiple quantum coherence type methods to generate the correlations. Only after the probe technology had substantially improved, and after we realized that in most cases in-phase single quantum coherence has favorable relaxation properties compared to heteronuclear multiple quantum coherence, did we shift to the use of more complex pulse sequences [27]. Subsequently, after Kay's ingenious introduction of gradient-enhanced coherence selection [28], these methods all have been adapted further to gain another $\sqrt{2}$ in sensitivity, while also greatly improving suppression of the water signal. Equally importantly, for larger perdeuterated proteins, TROSY-based gradient-enhanced versions have extended applicability of the approach to even larger proteins [29].

Novel approaches frequently sprout up simultaneously and independently in multiple laboratories. Triple resonance NMR is no exception in this respect. Gerhard Wagner and Guy Montelione, initially motivated by the desire to measure more accurate J couplings, developed 2D triple resonance experiments very similar in concept to ours [30]. This work coincided with Wagner's move from Michigan to Harvard Medical School, and if it were not for this interruption his laboratory would likely have extended the technology to 3D triple resonance of proteins in the same way as carried out by us at about the same time.

Today, the triple resonance 3D experiments have evolved into a streamlined suite of acquisition and analysis procedures, often executed in a largely automated "black box" manner. It is perhaps interesting to realize that much of this modern black box emerged in a largely iterative manner in which a large number of laboratories have played a critical role, starting from an initial array of gray and blue boxes (Fig. 2).

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