Bax, Ad: NMR of Ethanol and Interferon-γ

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GETTING STARTED

Ever since NMR was first discovered, NMR spectroscopists have been battling the technological problems that present the major barrier between creativity and experimental results. In the early 1970s, Toon Mehlkopf in the Applied Physics Department at the Delft University of Technology, The Netherlands, got so frustrated by the technical limitations of commercial NMR equipment that he decided to design and build one himself, the so-called 300-MHz project. The system included four home-built synthesizers and was controlled by a Hewlett Packard 21/M20 computer with 32 kbyte of memory. It was designed to be capable of both CW and pulsed NMR, including such important techniques as spin tickling, rapid scan correlation spectroscopy, DEFT and SEFT experiments, and a long list of other experiments that have long since been forgotten. In 1975, I was lucky to be accepted by Toon to work as an undergraduate student on the development of the pulse programmer and the data acquisition software of his spectrometer. Indeed, once completed in 1977, this spectrometer was capable of performing virtually any experiment. It also could acquire FIDs of up to 300 k Word at speeds of up to 45 kHz and average those to data stored in double precision on the disk. It also could execute pulse sequences of arbitrary length and complexity.

In the summer of 1977 when our group leader, Jaap Smidt, returned from a conference where he had heard Richard Ernst speak about two-dimensional NMR, our spectrometer was put to the true test. The main hurdle, it turned out, was for me to understand what Aue, Bartholdi, and Ernst were talking about in their historic paper.[1] After it slowly started to dawn upon me, it was relatively little work to write the required pulse program and data matrix transposition routine. About four weeks after Smidt's return, we had our first homonuclear J-resolved 2D spectrum.

When the next spring Mehlkopf and Smidt suggested that I continue to work for a Ph.D., I was excited about this opportunity, as well as by the idea that this would postpone my national service by at least four years. My only fear was that 'all experiments had already been invented', so what was I supposed to work on?

My first two papers were the result of a two-day trip to Zürich, where I visited the labs of Richard Ernst and Kurt Wüthrich. After Alex Wokaun very patiently but in vain had tried to explain to me the principles of multiple quantum NMR, I got into a heated debate with Kuniaki Nagayama, then a postdoc with Wüthrich, about obtaining absorption mode homonuclear broadband decoupled spectra. According to Nagayama, and he was right of course, one cannot get such a spectrum from a skewed projection of a homonuclear J spectrum because of the 'cross section projection theorem'. Back in the Netherlands, and still frustrated about having been 'set straight' by a biochemist, I came up with a way around this theorem: constant time spectroscopy. By keeping the time between the initial 90° pulse and the first detected data point constant and shifting the position of a 180° pulse [$90^\circ - (T - t_1/2) - 180^\circ - (T + t_1/2) -$ Acq.], I obtained a homonuclear decoupled spectrum of ethanol, and my first paper.[2] Little did I realize at that time how useful this silly idea would become in heteronuclear NMR of ^{13}C-enriched proteins, where it is now widely used to eliminate ^1J(C,C) couplings.[3]

Another esoteric idea that would later prove useful occurred to me after I had been wrestling my way through a stack of re- and preprints that Alex Wokaun had given me on my Zürich visit: pulsed field gradients. Clearly it should be possible to separate the different orders of multiple quantum coherence by means of pulsed field gradients. It took Toon Mehlkopf and electronics engineer Tom Tiggelman less than two weeks to build a pulsed field gradient device (with lock interruption) and implement it on the home-built 300 MHz spectrometer. Although the idea worked,[4] eddy currents induced by the pulsed field gradients were a serious problem—self-shielded gradients had not yet been invented—and discouraged me from further pursuing it. Nowadays, in protein NMR there is virtually no experiment left that does not significantly benefit from the use of pulsed field gradients with self-shielded coils.

OXFORD BLUES

When Ray Freeman, frustrated by an antiquated CFT-20, heard about Mehlkopf's 300-MHz project, he promptly offered him a job. Fortunately for me, Toon was unable to accept and he suggested that I might be interested in conducting part of my Ph.D. work with Ray in Oxford. The next three years were tremendously exciting for me. Not only is Oxford considered a Mecca for oarsmen—one of my favorite sports—but it also proved to be a very exciting place to live, with long-reaching consequences for my future social life.

Ray's brilliant insight in NMR and his enthusiasm for science tremendously stimulated his research group and convinced me that science could be very enjoyable indeed. The coffee and tea breaks, which typically would take at least an hour, were the highlights of the day, to the great frustration of Gladys, the cafeteria manager, who never managed to close early and go home. My stay in Oxford coincided with that of Malcolm Levitt, Gareth Morris, Tom Frenkiel, David Max, Stewart Kempsell, A. J. Shaka, and James Keeler. Needless to say, that this was a most inspiring working environment.

When I arrived in Oxford, Stewart had just come back from a conference where he had seen organic chemists measuring ^{13}C–^{13}C coupling constants from the satellites in a ^{1}H-decoupled ^{13}C spectrum. There should be a better way to do this he argued, and as I was considered to be 'the' multiple quantum expert from my previous pulsed field gradient multiple quantum work, I was asked whether multiple quantum somehow could help. And that was how the INADEQUATE experiment was born.[5] Although the concept of multiple quantum filtration was developed independently by the Ernst group,[6] the INADEQUATE experiment stole the limelight, not in the least because of the acronym that Ray had come up with, which stands for Incredible Natural Abundance

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Double Quantum Transfer Experiment. It was not clear at that time that the extemely lengthy 128-step phase cycle that we proposed was doing nothing else but eliminating the 'T_1 memory' of the spins. Because the delay between scans was not much longer than the ^{13}C T_1, the z magnetization of the next scan would 'remember' the rf phases used in the preceding scan and therefore cancellation of the signal from isolated ^{13}C nuclei was not complete. By sheer coincidence, the order of the steps in the 128-step phase cycle would cause such effects largely to cancel.[7] Unfortunately, our complex phase cycle led to the idea 'the more phase cycling, the better—no questions asked'.

THE MILE HIGH CLUB

On the recommendation of Ray I went on to do postdoctoral work with Gary Maciel in his 'Mile High Proton Enhanced Nuclear Induction Spectroscopy' laboratory in Colorado. I could not have made a better choice. Gary was extremely enthusiastic and supportive of my rather crummy attempts to apply some of the high-resolution ideas in solids, none of which happened to work out too well. I was lucky to be in Gary's group at the same time as Nick Szeverenyi, who had a true fascination with building gizmos. My original prototype of a magic angle hopping device consisted of rubber bands and Q-tips and required the operator to yank twice every scan on a nylon string. Nick's design and construction of a stepper-motor driven hopping probe made the experiment actually work.[8] Another one of his challenging accomplishments at that time was the design and construction of a 'magic angle flipping' probe[9] which would alter the orientation of the rotor axis between the magic angle and the xy plane, twice per scan. The anticipated problem, to keep the spinner going, posed no real difficulty for Nick. The main problem turned out to be that after every 100 000 flips or so, the leads connecting the coil to the tuning capacitors would succumb to fatigue. This 'flipping probe' allowed us to do a 2D experiment where the isotropic ^{13}C shifts are detected during t_2 and the associated CSA powder pattern, scaled by $-\frac{1}{2}$, in t_1. The first powder patterns of dimethoxybenzene measured this way were highly distorted and exhibited some very unusual nonpowder pattern features. In search of the cause of the bizarre appearance of these patterns, I desperately—Waugh, forgive me—even started questioning the generality of 'coherent averaging'. Only by chance did we finally discover that these distorted patterns would turn 'normal' if one packed the spinner very tightly; otherwise the centripetal force of the spinner would cause a nonrandom orientation of the individual crystallites inside the rotor.

While I was studying the principles of solid state NMR, next door in the NMR Center Richard Griffey was trying to correlate ^1H and ^{15}N chemical shifts by selective coherent ^{15}N decoupling and observing the collapse of the imino proton doublets in ^{15}N-enriched tRNA. When Bruce Hawkins, manager of the NMR Center, mentioned this to me I knew there should be better ways to do this. Although in principle Geoffrey's 'Overbodenhausen experiment' (aptly named that way by Ray Freeman during one of our tea breaks, but now better known as heteronuclear single quantum correlation, or HSQC) would permit ^1H detection of the ^{15}N resonances,[10] an additional problem was posed by the intense H_2O solvent signal; solvent presaturation was not possible because of rapid imino proton exchange. Heteronuclear multiple quantum coherence (HMQC) solved this problem and allowed us to do the experiment using only a single ^1H pulse which was adjusted to have a null in its excitation profile at the H_2O frequency. After discussing the pulse scheme with Bruce, he built an inverse ^{15}N channel literally overnight and the next day we obtained a 2D ^1H–^{15}N correlation spectrum, first try! Even in retrospect this experiment, conducted on an old 360 MHz Nicolet spectrometer and using only 3 mg of a 25 kD macromolecule in H_2O, was quite impressive.[11] Although the same HMQC pulse schemes, together with a large number of alternative schemes, were proposed independently by the Australian group of Bendall, Pegg, and Doddrell,[12] the application of our experiment to a biological macromolecule and to natural abundance peptides was probably the main reason for the surge in popularity of these HMQC experiments. For me it was my first encounter with a biological application of NMR, and I liked it.

WELCOME TO THE GOVERNMENT

Although I loved Colorado, besides Gary Maciel none of the faculty at Colorado State were particularly impressed by my NMR experiments and I had to look for employment elsewhere. To my honest surprise, the National Institutes of Health offered me a tenure-track job. How on earth they could imagine that NMR, particularly the type of work I was known for, could benefit the NIH program was totally beyond me. Obviously, Ted Becker (the editor of this volume), Bill Eaton, and their colleagues at NIH had more foresight.

The first excitement occurred when Donald Davis, who had joined me for a sabbatical year, was trying to use Aksel Bothner-By's CAMELSPIN experiment[13] to measure NOE interactions under spin-locked conditions in a cyclic peptide that gave miserable spectra with the regular NOESY method ($\omega\tau_c \sim 1$). This CAMELSPIN experiment later became better known as ROESY, for rotating frame Overhauser enhancement (ROE) spectroscopy. Yes, for many proximate ^1H pairs in the peptide we observed beautiful ROE cross peaks with opposite phase to the diagonal peaks. However, there were also a number of cross peaks in phase with the diagonal, in apparent conflict with Aksel's predictions. Most notably, the geminal nonequivalent C^δ protons of a proline residue in the peptide showed a very intense positive cross peak. Considering the short distance between the two C^δ protons (1.77 Å) we had expected a very intense ROE cross peak for this ^1H pair, so why would it be of the wrong sign and have an intensity that exceeded that of the diagonal? It was my solid state NMR experience that tipped us off to the real cause of these anomalous cross peaks: they were due to homonuclear Hartmann–Hahn cross polarization.[14] Two resonances that feel the same spin lock rf field and that are J coupled should be subject to J cross polarization, just as in the heteronuclear case. Changing the offset and spin lock field strength indeed confirmed that these artifactual peaks were due to Hartmann–Hahn cross polarization. At the time, Donald and I suggested minimizing the Hartmann–Hahn artifacts by judiciously choosing the carrier position and the rf field strength.[14] A much neater solution was later proposed by

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A. J. Shaka, another Freeman pupil, which 'spin locks' the magnetization perpendicular to the rf field.[15]

Once we had recognized the origin of the Hartmann–Hahn artifacts, it was relatively straightforward to capitalize on them and use them for obtaining J connectivity. For maximum Hartmann–Hahn transfer the goal was to make the trajectories of the coupled spins as identical as possible. In retrospect therefore not surprisingly, our improved Hartmann–Hahn cross polarization schemes started to look more and more like heteronuclear decoupling schemes, where the objective is to make ^{1}H spins coupled to ^{13}C in the $|\alpha\rangle$ and $|\beta\rangle$ spin states undergo identical trajectories.[16] The fact that the chemical shift difference between two J-coupled protons is typically much larger than ^{1}J(C,H) makes the homonuclear Hartmann–Hahn (HOHAHA) cross polarization actually somewhat more demanding. Our initial attempt to publish the use of an MLEV16 mixing scheme was thwarted by a reviewer who argued, correctly, that the spectra had significant phase distortions and therefore were not all that useful. After a few more experiments it became clear that the artifacts were due to two distinctly separate effects. First, not only was the magnetization parallel to the y axis transferred from one spin to its coupling partner, but also the x-axis magnetization, albeit with a different efficiency. Second, even for a single noncoupled spin, for example the proton in chloroform, we found an offset dependent rotation about the z axis during the MLEV16 scheme, which at that time we naively attributed to pulse imperfections but which, as later shown by John Waugh,[17] is inherent to the MLEV16 scheme. In any case, both effects were easy to eliminate: trim pulses ensured that the x magnetization was effectively eliminated. Adding a 17th pulse along the y axis after each MLEV16 cycle caused the average Hamiltonian to be dominated by this term and transverse magnetization could no longer 'wander away' from y and, voila, MLEV17 was born.[18]

While trying to develop our homonuclear Hartmann–Hahn (HOHAHA) pulse schemes it became clear that the concept was not new. Lukas Braunschweiler and Richard Ernst had several years earlier proposed the use of isotropic mixing in the so-called TOCSY experiment[19] which accomplished magnetization transfer by means of a string of closely spaced 180 ° pulses. After its initial publication, this TOCSY experiment did not really 'catch on', probably because it did not work very well in practice. Although I am unaware of any detailed early studies on the efficiency of the TOCSY experiment, the general feeling at that time was that an experiment with so many 180 ° pulses should suffer from rf inhomogeneity and other pulse imperfections. However, the true reason for its poor performance can be seen by considering the string of 180 ° pulses as a homonuclear Hartmann–Hahn cross polarization. Due to the different offsets of the coupled protons, the rotation experienced by off-resonance protons is larger than that for on-resonance protons, causing a 'Hartmann–Hahn mismatch'. Although even now the original MLEV17 mixing scheme still finds widespread use, more efficient mixing schemes have since then been developed by A. J. Shaka[20] and others, whereas Richard Ernst and co-workers devised an elegant scheme to eliminate ROE effects from macromolecule HOHAHA/TOCSY spectra.[21]

PROTEINS ARE BIG

For the first few years at NIH, I was primarily interested in studying complex small molecules such as natural products and peptides. When Laura Lerner joined my group she convinced me to add carbohydrates to the list. However, when in 1986 Michael Summers and I had completed a detailed NMR study of coenzyme B_{12},[22] primarily thanks to our development of the HMBC experiment, I had the feeling that methodologically this area might be maturing and my interests started to shift from small molecules to proteins. I had always avoided proteins like the plague, not in the least because I was horrified by the idea of staring into a light box for many months in a row. However, when Dennis Torchia, also at NIH, had managed to over-express and purify isotopically enriched staphylococcal nuclease, a protein of about 17 kD, he easily talked me into trying some of our small molecule experiments on nuclease. Together with Dennis and a very enthusiastic postdoc, Vladi Sklenar, we actually managed to get some neat experiments done. However, the big step came in 1988 when Dominique Marion and Lewis Kay decided they wanted to do 3D NMR. Dominique had joked that he 'was tired of working on weekends' and wanted an experiment that would run for at least three or four days. Unfortunately for him, a full disk on our old Nicolet spectrometer needed to be unloaded every 7 h or so, days, nights, ... and weekends too. Right from the start it was obvious to us that heteronuclear 3D was the way to go and our first and successful attempt was to record a 3D ^{15}N-separated NOESY spectrum.[23]

At that point it appeared to me that protein NMR might have a future after all and it was easy to convince NIH that investing in protein NMR would be a useful move. For starting a dynamic protein NMR program it would be essential to have a critical mass and we were fortunate to attract Marius Clore and Angela Gronenborn to the NMR program, which formed the beginning of a close and very productive collaboration. We also managed to obtain a significant commitment from NIH for space and capital equipment. With the arrival of two new AM-type Bruker spectrometers we were set to go and make serious attempts at recording high quality 3D spectra. The first hurdle that needed to be overcome, however, was the Bruker itself. In the normal mode of operation the ASPECT computer would require up to about 10 s at the completion of each increment to recompile the pulse sequence and get going again. Considering that most 3D experiments require only very few transients per increment, this compilation time more than doubled the duration of our 3-day 3D experiment, causing us some serious headaches. Fortunately, Rolf Tschudin, our electronics engineer, hired away by Ted Becker from Varian some 15 years earlier, was able to solve our problem by designing and building a box that would interrupt the Bruker pulse programmer clock for a defined amount of time that would increment each time the 'Tschudin timer box' received a clock pulse. This invention greatly reduced the time needed for recompilation of the pulse sequence and made 3D into a practical approach on the AM systems.

TRIPLE FUN

After our initial excitement about being able to record high-quality ^{15}N-separated NOESY and HOHAHA spectra, reality

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sank in. Making complete resonance assignments based on these two types of 3D spectra was going to be a very long and tedious process. The protein calmodulin that my new postdoctoral associate Mitsu Ikura had just started to work on was probably the best possible example for illustrating how difficult it could still be to make the sequential assignment following the well-established Wüthrich procedure.[24] Because of the very high degree of α-helix in this protein, the amide and H^{α} chemical shift dispersion was particularly poor.

At that time, my colleague Dennis Torchia was exploring the possibilities for using several variations of the Kainosho double-labeling approach[25] for making sequential assignments. By selectively ^{13}C labeling a certain amino acid type, A, in the carbonyl position and another type of amino acid, B, with ^{15}N in the amide position, only B-type amino acids that are immediately preceded by an A-type residue show a $^{1}J(N,C)$ splitting in a $^{1}H-^{15}N$ shift correlation spectrum. However, complete backbone assignment with this double-labeling approach required a large number of sample preparations and therefore a lot of work.

Dennis had the foresight to order a triple resonance probehead for his system; one that was optimized for proton detection but that also had a larger doubly tuned $^{13}C/^{15}N$ coil. I remember it took quite some effort to convince Bruker that this 'crazy' probe was what we wanted. With the capability to record 3D spectra already available, it was a relatively small step to come up with the so-called HNCO 3D experiment. This experiment ideally requires uniformly $^{13}C/^{15}N$-enriched protein, so every backbone amide ^{15}N is preceded by a ^{13}C carbonyl and generates a spectrum where every backbone amide proton gives rise to a single resonance, with ^{1}H, ^{15}N, and ^{13}CO shifts determining the position of the resonance in the F_3, F_2, and F_1 dimension. The ^{13}CO shift itself is not very informative, but by conducting a second 3D experiment (HCACO) that correlates H^{α}, C^{α}, and the ^{13}CO shifts we argued it should be possible to correlate H^{α}/C^{α} of one residue with the $^{15}N/^{1}H$ resonances of the next residue, as both would connect to the same ^{13}CO chemical shift. Again we were fortunate to have Rolf Tschudin in our lab who was able to build very rapidly a third rf channel that we could control from the Bruker pulse program and actually try out these 'Gedanken experiments'.

Primarily due to the creativity and persistence of Lewis Kay, we were finally able to make the triple resonance experiments work, and using the new 3D data Mitsu Ikura was able to complete his backbone calmodulin assignments shortly thereafter. Of course, we were tremendously impressed by our own accomplishments and were anticipating rave reviews when we submitted our results to the journal *Biochemistry*. To the contrary, the paper[26] was initially rejected. Such 'complicated experiments' requiring the very expensive ^{13}C labeling were deemed to be of no practical interest!

The next step in our structural study of calmodulin required making the side-chain assignments of calmodulin. Unfortunately, the presence of ^{13}C needed to disperse the $^{1}H-^{1}H$ correlations into a third dimension also causes severe broadening of the ^{1}H resonances due to the strong $^{1}H-^{13}C$ dipolar interaction. Regular $^{1}H-^{1}H$ COSY approaches therefore were not expected to yield useful data for proteins the size of calmodulin and larger. Instead, we chose the same solution as for the backbone assignments: transfer magnetization from one proton to another via one-bond heteronuclear J couplings, from ^{1}H to attached ^{13}C via $^{1}J(C,H)$, from ^{13}C to a second ^{13}C via $^{1}J(C,C)$, and back to the ^{1}H attached to this second ^{13}C. Despite the fact that magnetization in this so-called HCCH approach is transferred by three steps instead of one, such experiments are very efficient because each of the three steps transfers via a coupling that is larger than the pertinent linewidth.[27] Independent of our work Stephen Fesik, Erik Zuiderweg, and co-workers came up with an analogous solution which relied on isotropic mixing of ^{13}C magnetization with a MLEV17 scheme.[28] Both approaches are currently widely used.

Staring at all his ^{13}C assignments, Mitsu noted that there appeared to be a correlation between C^{α} chemical shift and the protein backbone conformation: all $^{13}C^{\alpha}$ carbons in α-helical residues showed a downfield shift relative to their random coil position. A subsequent more systematic analysis of the C^{α} and C^{β} shifts in four different proteins by Silvia Spera clearly demonstrated the generality of Mitsu's observation.[29]

WE CAN COUNT TO FOUR

In the same way as the idea of 3D NMR had been circulating for a long time before its real use was actually demonstrated, extending the dimensionality of the NMR spectrum to four was an obvious step to make. However, rather than proving that we indeed could count to four, we were looking for an application that would demonstrate its use. It was clear that spectral crowding is most pronounced in NOESY spectra and initially we wanted to focus on $^{13}C/^{13}C$ separated $^{1}H-^{1}H$ NOESY, an experiment that later would prove to be extremely challenging.[30,31] Instead, Marius Clore had a better idea that was much easier to implement: separate the ^{15}N-separated 3D NOESY into a fourth dimension, ^{13}C. This could be done with a minimal amount of phase cycling and Lewis Kay was able to obtain an impressive 4D spectrum for Marius's interleukin-1β.[32] Subsequent attempts to obtain a high quality $^{13}C/^{13}C$-separated NOESY spectrum proved that this was far more difficult and although we finally did manage to obtain such a spectrum only after the introduction of pulsed field gradients has this experiment become routine.[33]

The performance of many of the triple resonance experiments was significantly improved by Stephan Grzesiek when we were trying to study the protein interferon-γ, which with a total molecular weight of over 31 kD presented a formidable challenge to our methods. Short T_2 values were 'killing' a number of the triple resonance experiments. By departing from our old dogma that 'a good pulse sequence must have few pulses', Stephan was able to improve dramatically the performance of many of them.[3] The concept of 'constant time' was reintroduced, allowing dephasing and frequency encoding to occur simultaneously instead of sequentially, and with a few additional pulses Stephan also minimized the time that magnetization would be in the fast-relaxing antiphase state. Stephan also introduced a number of experiments that J-correlate the amino acid side-chain resonances with the backbone amides which made it possible for him to simplify dramatically the assignment process and complete the interferon-γ backbone assignments.[34]

In the fall of 1991 Geerten Vuister, a graduate student of Robert Kaptein, joined my lab. Among other experiments, he

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developed a large number of ingenious schemes for measuring homonuclear and heteronuclear coupling constants, most of them based on the concept of what we refer to as 'quantitative J correlation'. In this approach, the experiment is designed in such a way that, for example, the intensity of a cross peak between nuclei A and B is directly related to their J coupling. This turns out to be very practical and widely applicable in protein NMR. For example, not only was Geerten able to use this approach for measuring J couplings between amide and H^{α} protons, which characterize the backbone angle ϕ, but he was also able to measure ^{3}J(N,C) couplings between side-chain $^{13}C^{\gamma}$ and backbone ^{15}N.[35] This latter coupling turned out to be very small, ~2.2 Hz for *trans* and ~0.5 Hz for *gauche*. Even in small molecules it had previously been difficult to measure such couplings, whereas with this new method we are now able to measure these values in proteins as large as 20 kD. Together with ^{3}J(C,C),[36] ^{3}J(C,N) defines the important χ_1 side-chain torsion angles and also aids in distinguishing the prochiral methyl groups in valine residues.

WHAT'S IN THE CRYSTAL BALL?

The introduction of stable isotopes in proteins by ourselves and others has extended tremendously the power of NMR for characterizing their structure. I anticipate that with further software development for automated assignment and for the analysis of the 4D NOESY spectra, determining a high-resolution structure for proteins up to a few hundred amino acids will become as routine as it is presently in crystallography. Undoubtedly, however, NMR will find increasing use in characterizing the dynamic features of a protein, which frequently are critical to its function and which may not easily be studied by other methods. The study of the dynamics of the linker region in calmodulin, carried out by Nino Barbato using the methodology that Lewis, Dennis, and I had developed earlier,[37] is an interesting example of such a study. In the crystalline state, the linker was found to be a rigid helix, giving the protein a dumbbell shape. In solution, at 35 °C and at physiological pH and salt concentration, five residues in the middle of the linker were clearly highly flexible as judged by their ^{15}N relaxation properties. Analysis of the relaxation rates for the amides in the small globular domains of the protein showed that these domains were subject to nearly isotropic rotational diffusion, in contrast to what would be expected for a rigid dumbbell.[38]

Thus, it came as no surprise when subsequently Ikura, with the help of Marius Clore and Angela Gronenborn, was able to show that upon binding to its target the two globular domains come together with the target clamped in between them.[39]

Present work is focusing on extending the size limit of proteins that can be studied by NMR by further improving the methodology. So far we have relied almost exclusively on ^{15}N and ^{13}C although it is clear that deuteration can also be tremendously useful.[40] An initial attempt by Stephan Grzesiek to combine both approaches showed encouraging results and yielded substantial line narrowing of the deuterated C^{α} carbons.[41] A large number of different types of approaches along similar lines can be envisaged and only time, and lots of experiments, will tell which ones will be of long-term value.

The prospects for protein NMR are roesy, equipment is no longer inadequate and there remains plenty of room for further development of crazed ideas.[42]

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