Kay, Lewis E.: Structure and Dynamics of Proteins – Big and Small

Lewis E. Kay
University of Toronto, Toronto, Ontario, Canada

EARLY HISTORY

My first big break in science occurred when Brian Sykes was recruited to the University of Alberta from Harvard in the mid-1970s. Several years later, I had the good fortune to join his lab as an undergraduate and I quickly became immersed in some of the nuances of 1 and 2-D NMR. My initial project involved writing a computer program to calculate NMR line shapes in systems undergoing chemical exchange. All that I can remember is that the code worked, could handle two or maybe three spins, and that shortly after it was written there was a disk crash and the program (which was not backed-up) was lost forever. Subsequently, as a senior in 1983, I began recording and analyzing 2-D spectra of small fragments of Ca^{2+}-binding proteins that were recorded in magnitude mode and represented the state of the art at that time. During this period, I had the great fortune of taking a quantum mechanics course from Ted McClung and a large section of the material was devoted to a density matrix treatment of 2-D NMR methods such as COSY, NOESY, and 2-D J-resolved spectroscopy. Ted was a fantastic teacher who had a deep understanding of the theoretical underpinnings of NMR and with his help I slowly became conversant in the wonders of spin-physics.

YALE DAYS

Having decided to pursue a graduate degree in NMR spectroscopy, I headed to Yale University where I worked in the group of Jim Prestegard. Jim is quiet, unassuming, and a real scholar, and the four years that I spent in his laboratory were extremely enjoyable. My first project, in 1984, is worth recounting briefly because it formed the basis for my understanding of pulsed-field gradients which turned out to be useful in some of my early work when I moved to an independent position at the University of Toronto. Jim was interested in measuring diffusion constants of fatty acids in bilayers. Ian Armitage, in the Molecular Biophysics and Biochemistry Department across town, had a Bruker CXP 200 spectrometer equipped with a pulsed gradient unit that could deliver gradients of up to a few hundred gauss per centimeter in the z-direction. It seemed like the perfect setup to measure diffusion except that the gradient coil would break every hour or two – clearly I had to work efficiently! Because the diffusion of lipids in a membrane environment is relatively slow, I decided to focus on using a double-quantum scheme involving a pair of ^{19}F probes that were attached to the fatty acid.[1] It is straightforward to show that the double-quantum diffusion experiment is fourfold more sensitive to gradients than a single-quantum approach so that one could apply the diffusion technique to more slowly diffusing molecules than otherwise possible or, from a more practical perspective, one could use weaker gradients to minimize breakage. My excitement at having produced my first pulse sequence was somewhat tempered when I stumbled on a paper published in 1982 by Vold, Vold and coworkers[2] and, in 1983, by Zax and Pines,[3] showing the predicted improvements to the diffusion experiment when using multiple-quantum coherences. In retrospect I am sure that there are others who have been scooped by these groups too!

MULTIDIMENSIONAL NMR SPECTROSCOPY

In the mid-1980s, two-dimensional NMR spectroscopy was the rage and applications involved proteins that could be as large as 75 amino acids. Unlike many of my colleagues, I was unimpressed because it seemed like one spent months painstakingly analyzing dots to produce relatively low-resolution structures. Clearly, there had to be a better way. Fortunately for me, I joined Ad Bax's group at the NIH as a postdoctoral associate just at the right time to participate in the development of double- and triple-resonance multidimensional NMR. Ad's group, with important input from other members of the NIH team, designed a significant number of experiments.[4] Dominique Marion and I wrote a multidimensional data-processing package that was based on 2-D New Methods Research software that we were using at the time and together we recorded and processed ^{15}N-edited NOESY and TOCSY datasets.[5,6] Later on, when Mitsu Ikura produced a uniform ^{15}N, ^{13}C-labeled sample of the protein calmodulin, the experiments were extended to the triple-resonance variety – both 3-D and 4-D – where backbone nuclei were correlated in a variety of different permutations to facilitate the straightforward assignment of proteins in the 20-kDa molecular weight range.[7] Central to all of the work was the extremely talented Rolf Tschudin who designed a large number of little electronic boxes to expedite data acquisition. This was an extremely exciting period and I remain very grateful to Ad for allowing me the opportunity to participate. I should state, in passing, that other laboratories also made important contributions, including the groups of Montelione and Wagner[8] and Zuiderweg and Fesik.[9]

My interests have always extended beyond structure and Dennis Torchia, Ad, and I developed a set of ^{15}N-based relaxation experiments for monitoring backbone dynamics in uniformly ^{15}N-labeled proteins.[10] These experiments have turned out to be very popular, which I never would have predicted. They also turned out to be a bit tricky to get right, as in the initial versions I neglected to consider differential relaxation of the two ^{15}N multiplet components from cross-correlation between ^{15}N–^{1}H dipolar/^{15}N chemical shift anisotropy interactions. This taught me the importance of measuring relaxation rates using as many different approaches as possible and showing the consistency of the results, a lesson which was put to good use in our subsequent ^{2}H dynamics studies (below).

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BACK TO CANADA AND PULSED-FIELD GRADIENTS

After close to 4 years at the NIH, my scholarships had run their course and it was time to find a more permanent job. Solving the two-body job problem is never easy – especially when the two bodies are similarly trained – but thanks in large part to Ad and Dennis, Julie and I had a couple of offers to choose from. Toronto seemed more attractive in the early 1990s than Baltimore, and we were offered positions in separate institutions, allowing us to develop our independent careers. Toronto provided full access to a brand new 500-MHz Varian spectrometer and after a 6-week mini-sabbatical in Luciano Mueller's laboratory, where I learnt the nuances of Varian, I was ready to start. Self-shielded pulsed-field gradients were just coming into play and I was eager to see if I could put my previous experience using gradients to use. Gradients were already being utilized for coherence transfer selection in 2-D NMR experiments but the resulting spectra were at least a factor of $\sqrt{2}$, less sensitive than the corresponding datasets obtained with quadrature[11–13] based on the traditional methods of STATES[14] or TPPI.[15] Mark Rance and colleagues at Scripps had already shown that it was possible to preserve both sine- and cosine-modulated t_1 frequency components in 2-D spectra.[16] I reasoned that adding a selection gradient during the t_1 period really should not change anything except that modulation was now a function of both chemical shift and position in the sample. Thus, the Rance trick would work equally well for gradient coherence transfer selection so long as the initial dephasing was undone once the magnetization of interest was obtained at the end. The experiment worked beautifully, I thought; however, the reviewers' comments were somewhat less than encouraging since some felt that gradient-enhanced sensitivity refocusing would not be possible. Fortunately for me, Sunny Chan, who was handling the manuscript for the JACS was more convinced than the reviewers and the paper was accepted. At the same time, Christian Griesinger also published a very similar idea but with an application involving a HNCO experiment,[17] rather than a $^{1}H-^{15}N$ HSQC, and both groups showed that it is possible to record gradient-enhanced spectra with $\sqrt{2}$ increased sensitivity relative to "unenhanced" datasets.

It was long known that the utility of gradients in high-resolution NMR would extend beyond coherence transfer selection of the component of interest to include the suppression of artifacts arising from pulse imperfections. Indeed, homospoils had been used in high-resolution NMR applications, but long gradient recovery times prohibited their routine use. With this problem eliminated by shielding, gradients became extremely powerful, with some of their different useful applications summarized by Bax and Pochapsky.[18] It was immediately clear that gradients could be used judiciously for water suppression leading to the recording of high-quality H^{α}-detected spectra in H_2O and Ranjith Muhandiram and I developed a series of experiments for backbone and side-chain assignments[19] and for NOE measurements[20] on a single H_2O-based sample that proved to be useful.

THE MORE RELAXATION THE BETTER

Impressed with the popularity of the ^{15}N backbone relaxation experiments, I decided that the time was ripe for the development of robust methods for quantifying protein side-chain motions as well. Studies of methyl side-chain dynamics in myoglobin,[21] BPTI,[22] and M13 coat protein[23] had already been reported, suggesting that there were significant side-chain internal dynamics in these proteins. These early experiments were based on ^{13}C-observe measurements and it was clear that for the general applicability of the approach, improved sensitivity and resolution would be key. Linda Nicholson, Dennis, Ad, and I had already looked at Leu side-chain dynamics in staphylococcal nuclease, selectively labeled with ^{13}C at the Leu C^{δ} positions, using 2-D $^{13}C-^{1}H$ correlation spectroscopy.[24] It was clear from these preliminary experiments that relaxation was more complex in methyl groups than for amide ^{15}N spins. The complexities had been well characterized by Larry Werbelow and David Grant in their famous treatise on relaxation[25] and had also been discussed by Vold and Vold.[26] The three $^{13}C-^{1}H$ dipolar interactions in the methyl group interfere and there are dipolar–CSA interactions as well, leading to different relaxation properties for each of the ^{13}C multiplet components, especially in the macromolecular limit.[27] Moreover, relaxation during magnetization transfer elements in the 2-D experiments leads to a preferential selection of the slowly decaying multiplets and, unless care is taken, relaxation rates measured in ^{13}C- and ^{1}H-observe experiments differ.[28] Even in the small molecule limit there are issues, as shown by Art Palmer and coworkers.[29] I spent the better part of two years working on the problem as a postdoctoral fellow and, while there were some successes,[28] the ordeal convinced me that there had to be a better way.

The old adage "if you can't beat them join them" is certainly true when it comes to solution versus solid-state NMR and measurements of side-chain motions in proteins. The solids community had long used the ^{2}H nucleus as a probe of dynamics in a wide range of molecules, including proteins[30] and lipids,[31] and it was well known that the quadrupolar interaction dominates the relaxation of the deuteron. A disadvantage of the solids experiments as applied to proteins, however, is the poor resolution requiring essentially a separate sample for every labeled site. A few conversations with Dennis Torchia and Gitte Vold convinced me to try using the deuteron as a spin-spy probe of dynamics in proteins in the solution state in a way that would allow us to measure all sites simultaneously. Besides, I was tired of NMR of spin-1/2 nuclei and needed the challenge of something more complicated. Toshio Yamazaki and Ranjith Muhandiram in my group, together with Brian Sykes, developed experiments to measure ^{2}H T_1 and T_2 relaxation times in $^{13}CH_2D$ methyl groups of proteins that were fractionally deuterated using a magnetization transfer scheme, $^{1}H \rightarrow {}^{13}C \rightarrow {}^{2}H \rightarrow {}^{13}C(t_1) \rightarrow {}^{1}H(t_2)$, which optimized both sensitivity and resolution.[32] Later on, Oscar Millet and Nikolai Skrynnikov in my group developed further experiments that measured the relaxation properties of three additional coherences that are specific to spin-1 systems and they were able to cross-validate all of the five ^{2}H relaxation rates.[33] Over the years, side-chain methyl dynamics have been analyzed in over two dozen proteins using this methodology, as reviewed by Wand and coworkers.[34]

In the past decade, our efforts have turned toward studies of "excited protein states" that are invisible to the traditional tools of modern biophysics. As long as such "invisible states" are populated at 1 or 2% of the dominant ground state and exchange with the ground conformation on

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the millisecond (ms) timescale, they can be characterized by Carr–Purcell–Meiboom–Gill (CPMG) relaxation dispersion methods. In the late 1990s, Pat Loria, Mark Rance, and Art Palmer came up with an ingenious modification of the basic CPMG sequence for measuring ms exchange processes in macromolecules using coupled $^{15}N–^{1}HN$ spin systems as probes[35] and this opened up a wide range of protein applications. Our initial interest in exchanging systems was generated in a collaboration with Rick Dahlquist on a cavity mutant of T4 lysozyme.[36] Frans Mulder in the lab showed that many of the peaks in the amide spectrum of the protein were exchange broadened. The exchange properties were subsequently quantified using an experimental approach developed by Frans, in which variable numbers of CPMG pulses are given during a constant time delay, greatly accelerating data collection. Using additional methyl dispersion data recorded with an experiment developed by Nikolai,[37] Frans and my graduate student Tony Mittermaier were able to establish that the exchange process involved residues localized to the site of the cavity. Since these initial studies, new pulse schemes and labeling methodologies have been developed that greatly extend the utility of the CPMG approach for structural studies of excited states. A postdoc in my laboratory, Dmitry Korzhnev, devised a set of six experiments for quantifying ms exchange processes at backbone amide positions[38] and applied the methodology to protein-folding reactions involving the formation of a low-populated, on-pathway intermediate.[39] Building on the work of Rieko Ishima and Dennis Torchia who had developed experiments for probing exchange at ^{1}HN and ^{13}CO sites,[40,41] Patrik Lundstrom in the lab worked out efficient labeling schemes and the corresponding pulse sequences for measuring $^{1}H^{\alpha}$, $^{13}C^{\alpha}$, and $^{13}C^{\beta}$ chemical shifts of the excited state.[42] Finally, other lab members, Pramodh Vallurupalli and Flemming Hansen, showed that it is possible to measure residual dipolar couplings in invisible excited protein states using variants of CPMG dispersion schemes that quantify exchange in molecules with a small degree of residual alignment.[43] It is now possible to measure excited-state ^{15}N, ^{1}HN, $^{13}C^{\alpha}$, $^{13}C^{\beta}$, ^{13}CO, and $^{1}H^{\alpha}$ chemical shifts along with $^{1}HN–^{15}N$, $^{1}H^{\alpha}–^{13}C^{\alpha}$, and $^{1}HN–^{13}CO$ residual dipolar couplings that form the basis for the determination of structural models of these elusive conformers.[44] Clearly, the CPMG experiment has come a long way in the ensuing half century since its development!

BIGGER IS BETTER

The development of triple-resonance spectroscopy has had a profound influence on the study of proteins by solution-state NMR, but there are limitations. For proteins with molecular weights greater than about 30 kDa, sensitivity becomes a serious issue and deuteration is often necessary. The question is what levels of deuteration are optimal. On one hand, generating proteins with (near) complete levels of deuteration at aliphatic and aromatic positions is beneficial to the signal from the remaining amide protons, whose transverse relaxation times are increased. On the other hand, however, the loss of side-chain protons severely limits the number of available proton probes to quantify distances, a requisite step in structural studies. A good compromise is one in which highly deuterated molecules are produced with protonation confined to specific methyl sites. This approach permits the recording of triple-resonance experiments with high sensitivity, with the ensuing measurement of methyl–methyl, methyl–NH, and NH–NH NOEs for structure elucidation. I posed the problem to Mike Rosen and Kevin Gardner in the lab and suggested that they figure out how to produce such an unusually labeled protein since it was clear that it was not an "off-the-shelf item from Sigma". Mike worked out a procedure involving expression in $^{2}H_2O$ using protonated pyruvate as the ^{13}C source and while deuterated, methyl-protonated proteins were generated, to our disappointment the samples contained all the possible methyl isotopomers.[45] Kevin then came out with a beautiful solution, using precursors that would deposit $^{13}CH_3$ methyls at the γ and δ methyl positions of Val and Leu, respectively, and at the $\delta 1$ position of Ile.[46,47] Initially, we had to produce the Ile precursor biosynthetically ourselves, starting from labeled Thr, but eventually we managed to convince both CIL and ISOTECH of the profound financial gains that would await them if they made the compounds for the NMR community at large. Kevin then proceeded to show the utility of the labeling approach through detailed studies of maltose-binding protein, a 42 kDa, 370 residue protein[48] and subsequently Geoff Mueller was able to produce a well-defined global fold of the molecule.[49] The real breakthrough came when we put our approaches to the "test" by working on malate synthase G (MSG), an 82 kDa, 723 residue enzyme that seemed impossibly complex at the time. A graduate student, Peter Hwang, suggested the system and then developed a purification procedure before handing the project to Vitali Tugarinov who had recently joined the laboratory. Using Daiwen Yang's 4-D TROSY-based triple-resonance pulse schemes[50] Vitali recorded a series of assignment spectra on highly deuterated protein and with these he generated a very near complete set of assignments.[51] Subsequently, a global fold of the enzyme was obtained using a methyl-protonated, highly deuterated sample.[52] Along the way, Vitali and I invented many new pulse sequences, but perhaps the most exciting work was done on Christmas Day 2002, when he and I noted that the signal to noise in HMQC spectra of a highly deuterated, Ile $\delta 1$-$^{13}CH_3$-labeled sample of MSG was far higher than in corresponding HSQC datasets. This difference could be explained by considering the relaxation properties of the individual methyl multiplet components along with the magnetization flow in each of the pulse schemes and is the result of a methyl–TROSY effect where dipolar cross-correlation interactions efficiently cancel for half of the signal.[53] The very significant gains in sensitivity and resolution immediately suggested that NMR studies could be extended in a quantitative way to protein systems with molecular weights in the MDa regime. Indeed, Remco Sprangers was able to exploit this TROSY effect in studies of the ClpP protease[54] (300 kDa) and the proteasome[55] (700 kDa), and Al Velyvis has carried out similar analyses on the enzyme aspartate transcarbamoylase[56] (300 kDa) in collaboration with Howard Schachman at Berkeley.

THE FUTURE

It is nearly impossible to predict where the next advances will emerge in the evolving NMR field. Certainly, in my laboratory, there is tremendous excitement about the possibility

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of determining structures of invisible, excited states that play critical roles in biochemical processes but that have been recalcitrant to study using more established methodologies. Presently, we are on the cusp of achieving this goal – at least in some cases. NMR studies of supramolecular machines promise to "fill in the gaps" that remain even after detailed X-ray or cryo-EM studies, with the hope of relating structure to function through quantification of molecular dynamics that must be critical for the activities of these molecules. Finally, on a more personal note, I am most deeply indebted to a very talented group of students and postdocs whose hard work and abilities have shaped my scientific past and present and whose unique insights and creativity will help shape its future.

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Biographical Sketch

Lewis E. Kay. *b* 1961. PhD Molecular Biophysics, Yale University. Postdoctoral work at the National Institutes of Health (with Ad Bax). University of Toronto, 1992 to present, currently Professor of Molecular Genetics, Biochemistry, Chemistry and Physics, 320 publications. Current research specialty: biomolecular NMR.

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