Published in final edited form as:

Arch Biochem Biophys. 2017 August 15; 628: 3–16. doi:10.1016/j.abb.2017.05.003.

Biomolecular NMR: Past and future

John L. Markley* and William Milo Westler

National Magnetic Resonance Facility at Madison, Biochemistry Department, University of Wisconsin-Madison, Madison, WI 53706, USA

Abstract

The editors of this special volume suggested this topic, presumably because of the perspective lent by our combined >90-year association with biomolecular NMR. What follows is our personal experience with the evolution of the field, which we hope will illustrate the trajectory of change over the years. As for the future, one can confidently predict that it will involve unexpected advances. Our narrative is colored by our experience in using the NMR Facility for Biomedical Studies at Carnegie-Mellon University (Pittsburgh) and in developing similar facilities at Purdue (1977–1984) and the University of Wisconsin-Madison (1984–). We have enjoyed developing NMR technology and making it available to collaborators and users of these facilities. Our group's association with the Biological Magnetic Resonance data Bank (BMRB) and with the Worldwide Protein Data Bank (wwPDB) has also been rewarding. Of course, many groups contributed to the early growth and development of biomolecular NMR, and our brief personal account certainly omits many important milestones.

Keywords

Biomolecular NMR history; NMR instrumentation; Computational approaches; Sample preparation and labeling; Pulse sequence development

Our early experience with NMR

John Markley grew up in Colorado and was a chemistry major and member of the ski team at Carleton College (Northfield, Minnesota). John started graduate school in Pharmacology at Harvard in 1963, but his friend from Carleton, Donella Hager (Meadows), who had graduated the year before and joined the Harvard Biophysics Program with Oleg Jardetzky, extolled the wonders of NMR and convinced him to switch to this graduate program and thesis supervisor. Just after John joined the lab, Oleg left on a one-year sabbatical. His parting advice to John was to have a look at polyamino acids. Donella was investigating histidine titration curves in ribonuclease A by NMR, so the two joined forces on both projects.

John's reading of the scant biomolecular NMR literature of the time showed clearly that early studies had established a strong basis for the field of biomolecular NMR. These investigations included NMR studies of the building blocks of proteins [44] and nucleic

*Corresponding author. jmarkley@wisc.edu (J.L. Markley).

acids [42], the publication of the first ^1H NMR spectrum of a protein (40 MHz spectrum of ribonuclease) [107] and a follow-up paper that interpreted some of the signals in terms of what was known at that time of the spectra of amino acids [43]. A subsequent study showed that the NMR spectra of a native and denatured protein were very different [51]. Another influential paper demonstrated that NMR signals could be resolved from individual protons in individual residues (histidine) of a protein [65].

A singular benchmark in John's early graduate student education was his attendance at the second meeting of the International Council on Magnetic Resonance in Biological Systems (ICMRBS) held at the Wenner-Gren Center in Stockholm in 1966. The honorary chair of the meeting, Hugo Theorell (who received the 1955 Nobel Prize in Physiology or Medicine for his discoveries concerning the nature and mode of action of oxidation enzymes), ended his opening remarks at the meeting with the following statement, "*At the end of World War II two immensely important contributions to the arsenal of methods for studying magnetic phenomena were made: Zavoiski described the electronic paramagnetic resonance, and Purcell and Bloch the nuclear magnetic resonance. A great part of this symposium will be devoted to results obtained by these methods, that have introduced a new branch of science: The 'submolecular biology' [29].*" The first speaker, William D. Phillips (from DuPont), began his presentation with, "*Elucidation of the structures and interactions of proteins and nucleic acids in solution is of central importance to the development of molecular biology. A number of techniques are available for such investigations, prominent among them being measurements of optical density, optical rotatory dispersion, and ultracentrifugation. None of these techniques is capable of revealing detailed information at multiple atomic sites of a molecule or interacting system. Nuclear magnetic resonance (NMR) spectroscopy possesses in principle such resolution [90].*"

The published proceedings of this symposium give a detailed snapshot of the status of biomolecular NMR as it was 50 years ago [29]. Many of the presentations were from physicists applying their background in the theory of NMR, EPR, and ENDOR to model biological systems. William D. Phillips showed the first 220 MHz NMR spectra of proteins at the meeting, and demonstrated the resolution of high-field methyl signals from Ile, Leu, and Val residues attributed to ring current effects from aromatic side chains; Harden M. McConnell reported effects of spin labels on the NMR spectra of proteins; Mildred Cohn and co-workers reported line narrowing of NMR solution spectra of polymers by magic angle spinning; Martin Saunders discussed studies of fast reactions monitored by NMR lineshape analysis; Helmut Beinert and William H. Orme-Johnson presented their studies of EPR relaxation as a probe for active centers of paramagnetic enzyme systems; J. Bearden and T. H. Moss discussed the use of Mössbauer spectroscopy in studies of iron-containing proteins; and A. Hedberg reported the use of the Fourier transform in resolution enhancement of EPR spectra [29]. John's contribution to the meeting, which didn't make it into the printed proceedings, was the finding from 60 MHz NMR studies of helix-coil transitions in polyamino acids that the upfield shift of the $^1\text{H}^\alpha$ signal is diagnostic of helix formation [66]. 1966 also was the year that Richard R. Ernst and Wes A. Anderson published their application of Fourier transform spectroscopy to magnetic resonance [31].

Milo Westler grew up in Western Pennsylvania and attended Grove City College (Grove City, Pennsylvania), where as a chemistry major he carried out undergraduate research in synthetic organic chemistry with John Shaw that resulted in a couple of publications. Excited by this work, Milo decided he wanted to go to graduate school. However, his professors were reluctant to write letters of recommendation for him to join a Ph.D. program because of his low grade point. Following their suggestions, he joined the M.S. program at John Carroll University (University Heights, Ohio), where he worked with Richard Gaul. Milo synthesized a number of penicillin derivatives, became quite efficient at obtaining IR spectra, and had his first introduction to a Varian 60 MHz CW NMR spectrometer. His compounds were not very soluble, and Milo was challenged to obtain good NMR spectra on a spectrometer with no computer averaging capability. This experience embedded NMR in his soul. In his last year at John Carroll, a representative from Purdue University Chemistry came to recruit graduate students for their Ph.D. program. Milo applied to Purdue and was accepted. After arriving on campus in 1974, Milo learned that someone named Markley did NMR and went to talk with him. John reluctantly took Milo into his group, and they have worked together ever since.

We describe below our view of the evolution of biomolecular NMR in terms of developments in (1) NMR instrumentation, (2) computers, software and computational approaches, (3) databases, nomenclature standards, and software, (4) sample preparation and labeling, (5) pulse sequence development, (6) correlation of chemical shifts with macromolecular structure, and (7) research themes. Advances in each category clearly influenced the others.

NMR instrumentation

The Oleg Jardetzky lab at Harvard Medical School (HMS), where John was a graduate student, had a Varian DP-60 NMR (60 MHz, 1.41 T) spectrometer with a resistive magnet located in the basement under the gross anatomy laboratory and perfumed by formaldehyde from above. The magnet was “shimmed” with a lever that altered the alignment of the pole caps, and the magnet stability varied with the current in the mains. The arrival of Boston MTA trains three blocks away disrupted spectra, and when the lights went on for a home game at Fenway Park, one simply called it a night.

The lab moved to the Merck Institute (Rahway New Jersey) in 1967 (Fig. 1), where John completed work on his Ph.D. thesis under the official Harvard mentorship of Elkan R. Blout. At Merck, the group acquired a Varian HA-100 (100 MHz, 2.35 T), whose resistive magnet was equipped with a “superstabilizer” to control the field. The spectrometer was completely enclosed in a housing in an attempt at improved temperature control.

The state-of-the art at that time was the Varian 220 MHz (5.17 T) NMR spectrometer, the first to be equipped with a superconducting solenoid. The first two of these spectrometers were in industrial labs, one at DuPont and the other at Bell Labs. Bob Shulman at Bell Labs graciously made his spectrometer available to John for acquiring spectra of selectively deuterated staphylococcal nuclease [67]; the 2.2-fold increase in resolution over the Merck instrument was astounding. The curious feature of the Varian 220 was that the probe was

fixed, and one raised the superconducting solenoid, which was counterbalanced by a weight, to change samples.

John chose to work with Melvin P. Klein, in the Melvin Calvin Chemical Biodynamics Laboratory at UC Berkeley, starting in 1970 under his NIH postdoctoral fellowship, because it had a Varian 220 (Fig. 2) and was the first academic lab in the US with (homebuilt) FT-NMR capabilities (on a 60 MHz NMR spectrometer). John used this instrument to develop the saturation-recovery approach to T_1 measurements [70]. The spectrometer was highly modular and needed to be physically rewired to change from one pulse sequence to another. One of the first things John did was to create wiring diagrams for key experiments. Although FT was promised on the Varian 220, this was realized only after John had left the lab for Purdue. A subsequent advance in NMR instrumentation was the Varian 300; this spectrometer never became popular, because it was not FT-ready (even though the technique was developed at Varian) and was outclassed by the Bruker 270 MHz NMR spectrometer with its FT capability, which we tested for one of our early ^{13}C studies [81].

The Purdue Chemistry Department, which John joined in 1972, had a Varian XL 100-15 spectrometer equipped with an internal deuterium lock. Bob Santini, our instrumentation guru, subsequently added a home-built Fourier-transform modification based on a Nicolet 1080 computer, which we found useful for ^{13}C and ^{31}P NMR spectroscopy of proteins [4,97].

For high-field NMR, we were fortunate in the early 1970's to become regular users of the NMR Facility for Biomedical Studies at Carnegie-Mellon University (CMU). For a short period, non-stop jet service linked the Purdue University Airport with Pittsburgh, and John and his group members were loyal passengers. At CMU, they carried out correlation spectroscopy on the home-built 250 MHz NMR spectrometer constructed by Joe Dadok and Aksel Bothner-By. The experiment utilized cross-correlation of a continuous wave spectrum recorded in fast passage with a single reference line recorded under the same conditions [22]. The fast scans toward, but stopping short of, the water signal were time averaged and then deconvolved against the reference spectrum to remove the phase wiggles. This proved to be an excellent approach for observing signals from histidine residues and aromatic residues in proteins dissolved in D_2O [74,76–80,82,83,96]. In December 1979, the CMU resource developed the World's first 600 MHz spectrometer [11]; the magnet was non-persistent and utilized active field regulation. The availability of these spectrometers and the excellent support from the CMU resource was a key factor in John's achieving tenure at Purdue.

John founded the Purdue University Biological Magnetic Resonance Laboratory (PUBMRL) in 1977 with a P41 grant from the National Center for Research Resources, NIH. The grant enabled the purchase of a wide-bore Nicolet NT-150 (3.52 T), later upgraded to an NT-200 (4.68 T), and a Nicolet NMC-360 (8.46 T), subsequently upgraded to an NT-470 (11.04 T) spectrometer (Fig. 3). When PUBMRL's Operations Manager, Jerry Dallas, decided abruptly to move back to California in 1979, Milo, while still a graduate student, was pressed into service as his replacement. In the lab, Milo focused on studies of H-bonding in serine proteases, an area pioneered by Robillard and Schulman [102–105]. Milo contributed

to this project [84,97], which involved collecting protein NMR spectra in water ($\sim 100\text{ M }^1\text{H}$). Milo's reading of the literature suggested that the "Redfield pulse" [100], similar to a long pulse, could be used to suppress the huge water signal without saturation, which would allow the detection of the 1 mM signal arising from the H-bonded proton of histidine. Milo applied this approach to studies of the H-bonded proton in alpha-lytic protease [127]. He was surprised that setting the pulse width to the reciprocal of the offset he wanted to suppress, gave a HUGE signal from the water. Something was very wrong. The early Nicolet spectrometers were fitted with a sound channel so that the user could hear the FID. One could put a 99.9% D₂O sample into the spectrometer and set the frequency some distance from the water signal to give a pleasant "tone" from the residual hydrogen atoms and then manually adjust potentiometers attached to the 36 shim channels to get a long lasting booooooooooooooooooooo. It was a great way of getting good homogeneity. Milo used this for shimming but found that it could also be used to determine solvent suppression as one adjusted a potentiometer to change the pulse width. The resulting pulse width that gave the best "suppression" (lowest amplitude) was nowhere near the value suggested by the Fourier transform. For Milo, this was the beginning of his use of NMR theory to figure out what was going on. His interest in theory (NMR, quantum mechanics, MD, etc.) grew from there.

Following our recruitment from Purdue to the University of Wisconsin-Madison in 1984, we founded the National Magnetic Resonance Facility at Madison (NMRFAM). Our first spectrometers were a Bruker wide-bore 400 MHz (9.39 T) and 500 MHz (11.74). A second 500 MHz spectrometer was on loan for a promised 600 MHz (14.09 T) spectrometer, which was delivered in May 1988 (Fig. 4). The next spectrometer, a Bruker 750 (17.62) delivered in 1994, was too large for the current NMR facility and had to be housed a mile distant in temporary quarters until the completion of a new NMR lab in 1998. A Varian 800 (18.79 T) was delivered in 12/2001 and a Varian 900 (21.14) in 7/2004 (Fig. 5). Fig. 6 shows the dates of delivery and field strength (in ^1H frequency) for the NMRFAM spectrometers.

Computers, software, and computational approaches

Spectral "averaging" in John's early days at Harvard in the mid 1960's, consisted of superimposing continuous wave scans in each direction and averaging by eye to determine peak positions. Peak positions were determined by using a ruler to interpolate between markers of known frequency injected by a frequency synthesizer on either side of the scan. The Jardetzky group at Harvard Medical School acquired a "computer of average transients" (CAT) and adapted it for NMR spectroscopy [45]; the CAT was a device devised originally for recording physiological responses. While offering a step forward, the output of the early CAT not available in digital form; it was viewed on an oscilloscope and could be photographed. Early NMR spectrometers utilized flatbed recorders for printing spectra; the Achilles' heel was the pen, which utilized India ink, and was subject to clogging. Some models would shake the pen before printing to increase the chance for success.

The Jardetzky lab's Varian HA-100 NMR spectrometer at Merck was interfaced with a Varian C-1024 computer of average transients; the output could be fed to a computer card punch for data storage and subsequent analysis. Peak calibration still required a complicated procedure [68]. At Merck, John had access to a DuPont 310 Curve Resolver, an analog

computer that enabled the simulation of a series of overlapped Gaussian/Lorentzian lines. A bank of potentiometers (one for each peak) controlled the peak position, width, and intensity for each peak; once a good fit was obtained, the individual components were printed, cut out, and weighed to determine relative intensities. In Mel Klein's lab at UC-Berkeley, we recorded spectral data from the 60 MHz NMR spectrometer on magnetic tape. We then used the lab car to transport the tape to the Lawrence Berkeley Laboratory, where we submitted it along with a computer card deck with control cards specifying the program to be run and a guess at the phasing parameters.

Data acquired at the NMR Facility for Biomedical Studies at CMU in Pittsburgh were processed on a Sigma 5 mainframe computer (Scientific Data Systems). This system, which consisted of five full-size cabinets with a monitor, control panel and a printer was donated in 2002 to the Computer History Museum located at NASA's Ames Research Center in Mountain View, California. (http://www.computerhistory.org/VirtualVisibleStorage/artifact_frame.php?tax_id03.02.03.00).

In the early 1970's our slide rules became outmoded by small handheld calculators culminating with fancy programmable HP models. John's first desktop computer was a Heathkit that he assembled himself. The Varian XL 100-15 at Purdue initially was CW-only with spectra stored on paper tape. Later, Bob Santini modified this spectrometer for FT-NMR by adding a home-built pulsing system and a Nicolet 293 I/O unit with a Diablo Model 33 disk drive. When we finally acquired funds for spectrometers with superconducting solenoids at Purdue, we chose Nicolet (a Madison, Wisconsin, company) because of its advanced computer and its capability of supporting both fast-scan correlation spectroscopy and FT NMR. The early computer graphics systems that we used were nearly as expensive as a low-field NMR spectrometer. These dedicated computer graphics systems and the specialized computers on NMR systems were eventually surpassed by commodity desktop and laptop computers. Before the advent of digital printing, we added lettering to illustrations by hand using templates, India ink pens, and lots of white-out correction fluid. The "Photo Shop" was not a computer program, but instead was the place where the hand-annotated diagrams were converted to 8" × 10" glossy prints for journal submission.

While the Nicolet computer systems at PUBMRL were a great advance, they suffered limitations with respect to pulse sequence development, especially given the rapid advance of NMR pulse sequences during the 1980s. The Nicolet pulse programmer was limited to a total of 16 steps including any phase cycling. For the early multidimensional experiments, which were usually carried out in magnitude mode, this was sufficient, but trying to obtain phase sensitive COSY spectra on an instrument like this, required great effort to be able to squeeze the pulse sequence into 16 steps. Collection of the phase sensitive data also requires separation of the real and imaginary data components in order to retain phase sensitivity. This ended up requiring assembly code programming on the Nicolet computer.

The Bruker AM consoles acquired by NMRFAM in the mid-to-late 1980s, made the collection of 2D spectra significantly easier. However, issues arose when we started performing triple-resonance experiments, which required a third RF channel. The AM consoles only had two RF channels, but Ed Mooberry, our instrumentation specialist, was

able to procure the equipment (synthesizers, switches, splitters, amplifiers, lots of RF cables and BNC connections, etc.) needed to build a third channel. This equipment was controlled by the TTL levels from the pulse program coming out of the back of the AM console. This allowed the performance of ^1H , ^{13}C , ^{15}N experiments.

Another advance in NMR came with the introduction around 1990 of 3D NMR techniques [47]. The designers of the AM consoles had not foreseen this technology. However, in their wisdom, they supported the use of a macro language that could mimic all of the command-line inputs to enable procedures unsupported by the pulse sequence language. The console computer permitted access to a Pascal program compiler. The concept for obtaining 3D spectra is only an extension of the method for obtaining 2D spectra, so it was easy to increment a third dwell time during data collection by means of a macro script written in Pascal. Two-dimensional spectra were collected and then the macro would increment the third dwell time and start a new 2D data collection. The bigger problem was in data processing as there was no capability for processing the third dimension in Bruker software. Zsolt Zolnai proceeded to write a Pascal program that ran on the AM consoles that would Fourier transform and phase the data in the third dimension once the first two dimensions were processed using standard Bruker software. Unfortunately, the processing of 3D data took overnight or longer, which tied up the off-line data processing console. As part of another project, Zsolt had purchased an array processor (Fig. 7) that fit into the Windows desktop computers of the day. Unfortunately, Zsolt had to leave the lab to serve his required time in the Yugoslav military. That left Milo to write code for processing 3D spectra in Pascal and in the assembly code routines supplied with the array processor. The result reduced processing times to about 10 min.

The newer NMR consoles from Bruker and Varian arriving in the mid 1990s incorporated standard commodity desktop computers. Both vendors utilized Linux as the operating system, but Bruker also offered a Windows version. In addition to the capability of processing 3D NMR data with a button push, these computers enabled more facile connections between the NMR spectrometers and other software programs. NMRFAM's ADAPT NMR strategy [3], which connects frequency-domain, non-uniform sampling data collection [52] with spectral analysis for assignment of small to moderately sized ^{13}C - ^{15}N labeled protein systems, utilized this capability.

These days, spectrometer software has advanced to the point that almost every part of the hardware is easily accessible in the pulse sequence along with macro software. With freely available software like NMRPipe [23] along with inexpensive multiprocessor computers, it is almost a push-button environment to process and analyze data. The continuation of UCSF Sparky NMR analysis software through NMRFAM-Sparky [53] and other software makes multidimensional NMR data analysis rather routine. The major problem is the installation of these packages and their required libraries is often on Linux systems, which are generally not the first choice for most users of NMR these days. For several years, we have been using virtual machine technology to simplify the installation of software. VirtualBox is a cross-platform, freely available system that can easily be installed on almost all operating systems. Then a virtual machine (VM), with the software installed by Linux gurus, can be installed into VirtualBox and then used on any computer system desired [54]. NMRFAM is also

currently collaborating with the NMRBox project (<http://connjur.uchc.edu/projects/nmrbox/>) to create VMs with whatever combination of processing and analysis software desired.

Databases, nomenclature standards, and software

In 1984, Eldon Ulrich, a long-term member of the laboratory, and John assembled a collection of assigned protein chemical shift for a review article [85]. This exercise made it apparent to us that it was time to think about a publicly-accessible database of biomolecular NMR parameters. John worked on this idea during brief sabbatical at the Protein Research Institute at Osaka University as the guest of Yoshimasa Kyogoku. Following his return to Madison, Eldon, John, and Kyogoku published their ambitious proposal for such a database in 1989 [120]. The proposed database was to contain information about type of molecule, its name and sequence, atom description, authors and citation, sample labeling pattern and solution conditions, and description of NMR experiments. Assigned NMR parameters to be archived included chemical shifts, coupling constants, relaxation rates, and cross-relaxation rates. Results derived from NMR studies were to include site-specific pK_a values, hydrogen-bonding maps, secondary structural elements, hydrogen exchange rates, ring rotation rates, correlation times, conformational equilibria, and atomic coordinates. John and Eldon submitted a grant request to the National Library of Medicine (NLM), a branch of the National Institutes of Health (NIH), for the establishment of a publicly-accessible repository of NMR data, which was funded in 1988. BioMagResBank, or BMRB, was the name they gave to the repository, although the more descriptive name used now is “Biomolecular Magnetic Resonance data Bank”. The initial implementation of BMRB was as a relational database. Through a discussion with Helen Berman, John learned about the dictionary-driven, self-defining STAR format [38–40] used by the small molecule crystallography community. Eldon evaluated a number of options for data representation. ASN.1 and XML were not human readable, but STAR was readable and its “save-frames” allowed for multiple sets of chemical shifts or other NMR parameters to be represented as separate tables. This led to the decision by BMRB in 1996 to adopt the STAR format [121] as defined by the NMR-STAR dictionary. The NMR-STAR format enables the collection of all the types of data envisioned in the initial publication [120], as well as having the flexibility to incorporate new types of data, such as solid-state NMR and SAXS, in response to developments in the field. BMRB adopted NMR-STAR well in advance of the conversion of the Protein Data Bank (PDB) to mmCIF (a subset of STAR lacking save-frames). In the beginning, information in BMRB was gleaned from the literature, but after developing a web-based deposition system, we encouraged direct submissions from authors. The *Journal of Biomolecular NMR* was the first of several journals to require BMRB deposition of NMR data. Through BMRB’s subsequent affiliation as a partner in the Worldwide Protein Data Bank (wwPDB) in 2007 [7], the deposition of chemical shifts and later restraint data became mandatory for NMR structures deposited in the PDB. BMRB faced a crisis when the NLM decided in 1997 to stop funding external databases and no other funding agency agreed to take on the project. Following an outpouring of support for the BMRB [89], the National Institute for General Medical Sciences agreed to consider a grant proposal, and our submission was successful.

The early monographs from Kurt Wüthrich [134,135] were instrumental in organizing the concepts and nomenclature of biomolecular NMR. Kurt also organized a group of scientists under the auspices of the IUPAC and IUB to prepare recommendations for the presentation of NMR structures of proteins and nucleic acid; this led to the 1998 publication in *Pure and Applied Chemistry* [87], which was reprinted in various biochemical and structural biology journals. The BMRB adopted these recommendations, but it took several years and prodding by BMRB before the PDB became compliant with its atom nomenclature. Some software developers have been even slower to adopt the IUPAC-IUB recommended atom nomenclature.

Recently, a group of software developers sanctioned by the wwPDB proposed an NMR Exchange Format (NEF) [35], a simplified STAR format for restraint data of restricted types. The BMRB has developed software to convert NEF to NMR-STAR so that authors can use NEF as a deposition format for restraints. NMR-STAR offers a more comprehensive way of representing restraints than NEF, and it will remain the archival format for restraints. Software for validating restraints submitted to the wwPDB will process the NMR-STAR version.

Sample preparation and labeling

The early NMR studies of proteins generally were of enzymes and protease inhibitors that one could purchase commercially. The ribonuclease A, hen egg white lysozyme, and staphylococcal nuclease used in our first NMR studies of proteins were from the Worthington Biochemical Company (Freehold, New Jersey) [91]. Many of these proteins were highly soluble up to 10 mM or more.

The objective of John's Ph.D. project, was to test the hypothesis of his research supervisor, Oleg Jardetzky (as disclosed by Oleg at an NMR meeting in Japan [46]), that the ^1H NMR spectrum of a protein could be simplified by selective deuteration so that otherwise overlapping signals could be resolved. Fully protonated staphylococcal nuclease (SNase) was produced from *Staphylococcus aureus* cells grown in a 500-L fermenter; the yield was about 0.5 g pure protein. The path to selective deuteration involved (1) preparing a mixture of ^2H -labeled amino acids, (2) feeding these plus selected ^1H -amino acids to *S. aureus* (which helpfully requires many of these amino acids), (3) harvesting cells, disrupting them and isolating a protein (staphylococcal nuclease), and (4) collecting NMR spectra for comparison with that from the unlabeled protein [67]. All of this could not have been accomplished without the resources of the Merck Institute. The deuterated amino acid mixture was provided by Merck Canada, and the large-scale preparations made use of the Merck Pilot Plant in Rahway, NJ. These were the days before DNA sequences of proteins, recombinant protein production, PAGE, and His-tags. The presence of the desired enzyme was detected by biochemical assays for nuclease activity, and the purity of the enzyme was determined by specific activity and amino acid analysis [98]. Amino acid analysis of the deuterated amino acid mixture revealed the absence of tryptophan, and ^1H NMR spectra of the amino acid mixture showed that back exchange from D to H had occurred during HCl hydrolysis of the perdeuterated protein. In addition to back exchange of alpha protons, $\text{H}^{\text{e}1}$ and $\text{H}^{\text{e}2}$ of Tyr, and the $\text{H}^{\text{e}1}$ of His had back-exchanged from D to H. Natural abundance Trp

had to be added, and thus the ^1H NMR spectrum of the aromatic region contained signals from only the Trp ring and the simplified Tyr and His rings. Single signals were observed from each Tyr ring, as expected for fast rotation on the chemical shift scale (Fig. 8). We did not find ring flips remarkable at the time, because the X-ray structures in those days were not highly refined.

One of the shocks of John's PhD studies was his finding SNase exhibited signals from four histidine residues, despite its published sequence from Chris Anfinsen's lab having only three histidine residues [118]. He telephoned the Nobel Prize winner with the news. Fortunately, Anfinsen had an easy answer. There were two known strains of *S. aureus*: SNase from the V8 strain with the published sequence had three histidines, but SNase from the Foggi strain had the substitution L124H as was later published [21]. John acquired the Foggi strain, isolated its SNase, and compared the spectra of the two strains (Fig. 9). This yielded what was probably the first protein NMR assignment by mutagenesis [68].

The group later made use of the differential exchange properties of His residues as a strategy for assigning their NMR signals [73]. Signals from multiple His residues of a protein dissolved in D_2O were followed by NMR to determine their relative exchange rates. Then a parallel experiment was carried out with water spiked with HTO, and His-containing peptides were isolated following trypsin digestion, and the level in tritiation determined by scintillation counting. This strategy was used in assigning the His signals from soybean trypsin inhibitor [78], turkey ovomucoid third domain [72], and ribonuclease A [74].

With ^1H NMR of proteins at natural abundance, the low hanging fruit were the high-field methyl signals and the low-field signals from histidine ring protons and their H-bonded amide protons. We focused on the latter signals in our early days at Purdue [75,84].

Early ^{13}C NMR spectra of proteins were acquired at natural abundance, sometimes with NMR tubes with diameters as large as 20 mm [1]. The interpretation of these spectra benefitted from early catalogs of ^{13}C NMR spectra of amino acids [41].

Joseph J. Katz and Henry Crespi at Los Alamos National Laboratory (LANL) had been pioneers in applying stable isotopes to NMR spectroscopy [19]. During his cross-country drive in 1972 from Berkeley to West Lafayette, Indiana, where he was to begin as an Assistant Professor at Purdue, John made a detour to LANL with the objective of learning how Nick Matwiyoff and his group there were cultivating algae on $^{13}\text{CO}_2$ for the production of ^{13}C -labeled compounds. The group was using ^{13}C -labeled metabolites to carry out early metabolomics studies [24]. After becoming settled at Purdue, John designed an "improved" rocker box, a flat Plexiglas box in an angle-iron frame with an axle and bearings that allowed it to be rocked gently back and forth. Adjustable banks of fluorescent lights scrounged from Purdue Salvage were arrayed above and below the box. The box was equipped with a built-in pH electrode interfaced to electronics that operated a valve to let in $^{13}\text{CO}_2$ when the pH rose above a set point, and closed the valve when the pH fell below a set point. As cells used up $^{13}\text{CO}_2$ the pH would rise, the valve would open to bubble in $^{13}\text{CO}_2$, which would lower the pH, and turn off the valve. John hired a fearless undergraduate student, Dirk Tea-garden, to construct the apparatus; and the Instrumentation

Facility in the Chemistry Department built the electronics. Once ready, the box was filled with water, inoculated with *Anabaena* 7120, and the lights and rocking mechanism were turned on. The cells, however, died. After some sleuthing, we discovered that some of the fittings used in the tank contained copper. After these were replaced, the system worked perfectly. We were able to isolate 30% ^{13}C -labeled *Anabaena* 7120 ferredoxin [15,93], plastocyanin [50], and cytochrome c_{553} [136] from the cells.

The first residue-specific ^{13}C labeling of a protein was carried out in 1973 by Browne and co-workers at UC Berkeley [12]. A further goal, of course, was to be able to label single residues selectively rather than all of a given kind. This had been done by chemical synthesis for the ribonuclease S peptide [14], and was an approach we used much later ourselves with rubredoxin [58]. Our colleague, Mike Laskowski, Jr., at Purdue, had developed a method for replacing the P1' residue of a protein proteinase inhibitor, by enzymatic semi-synthesis [108]. We adapted this procedure to place a ^{13}C -label at the reactive site amino acid residue of soybean trypsin inhibitor (STI). The procedure was to use a catalytic amount of the trypsin to cleave the Arg63-Ile64 peptide bond at the reactive site; then carboxypeptidase B was used to remove the P1' residue (Arg63). Next, ^{13}C -Arg was added to drive the carboxypeptidase B-catalyzed reaction backwards to resynthesize the Tyr62- ^{13}C -Arg peptide bond, and the addition of 1:1 trypsin led to resynthesis of the ^{13}C -Arg63-Ile64 peptide bond. Finally, the selectively labeled STI was released from trypsin by quickly lowering the pH. The goal of the experiment was to answer a provocative question at the time, namely, what is the state of the reactive-site peptide bond in the trypsin-STI complex. The initial conclusion from X-ray crystallography was that the bond in the complex was fully tetrahedral [117], but a later X-ray study suggested that it was only partially tetrahedral [9], our results from analysis of the ^{13}C chemical shift indicated that it was more likely trigonal, although we could not rule out partial tetrahedral distortion [4]. Our paper was published back-to-back with an independent, but similar, study of the trypsin-BPTI complex by Wüthrich and Tschesche [101].

Between 2001 and 2016, grants from the NIH Protein Structure Initiative enabled us to refine our approaches for protein production and labeling for NMR studies [34,88] and to develop a new platform based on wheat germ cell-free protein synthesis [6,64,122–124]. For human proteins, the two approaches appeared to be highly complementary [119].

Pulse sequence development

His first encounter with the Redfield pulse sequence for water suppression intrigued Milo. What was going on? During the remainder of his Ph.D., Milo enjoyed “playing” with pulse sequences and computations. His first computational approach came in the form of a Basic program that simulated the Bloch equations. This program ran on the Heathkit computer that John had built. He finally was able to understand the Redfield pulse sequence that stumped him earlier. Milo, while working on alpha-lytic proteinase, was interested in obtaining the pK_a value for the single histidine residue located at its active site. To simplify the histidine region that overlaps with the amide spectral region, exchange of the amide protons with deuterium is often used. Milo found that alpha-lytic proteinase was recalcitrant, and no conditions could be found to exchange the amide protons. Milo found a paper by Iain

Campbell [13] based on the early work of Hahn [36] that suggested the use of the spin-echo sequence would remove broad amide signals and allow the observation of the sharp histidine ring proton signals.

Two days after Milo obtained his Ph. D. in 1980, John left Purdue for a one-year sabbatical in Montpellier, France. This left Milo to his own wiles. He became further interested in spin-echo sequences and noted that the Campbell paper [13] demonstrated the well-known phenomenon that scalar couplings cause amplitude modulations of singlets and doublets [37]. He used this sequence to help identify different multiplicities in turkey ovomucoid third domain, a serine proteinase inhibitor [86]. This also lead him to the use of 2D NMR and the subsequent reading of Ad Bax's Ph.D. thesis [5], especially Appendix I, which contains details about the density matrices and rotation operators used to calculate the effects of coupling in NMR pulse sequences. Combined with the introduction of product operators by the Ernst group [113], this led to Milo's operational understanding of NMR theory.

The proton spin-echo experiments, led Milo to read Gareth Morris' paper on the INEPT sequence [92], which contained a spin-echo experiment between ^1H and ^{13}C . The vector pictures developed to describe these sequences were gripping for him. Milo always had an interest in rotations stemming from his days thinking about three-dimensional reactions in organic compounds, so this made sense. The INEPT sequence, in effect, transfers ^1H polarization to a coupled ^{13}C nucleus through ^1H - ^{13}C scalar coupling. Combining this with his newly found knowledge of two-dimensional NMR spectra, we were able to obtain ^{13}C -detected 2D ^1H - ^{13}C spectra of ^{13}C -labeled proteins, which were provided from John's wet lab efforts in growing the cyanobacterium *Anabaena* 7120 on ~30% $^{13}\text{CO}_2$.

The move to Wisconsin in 1984 and the founding of NMRFAM brought significant improvements in NMR instrumentation, allowing advancements in pulse sequence development: proton-detected heteronuclear capability, high-field sensitive, multinuclear probes, and the ability to carry out complicated pulse sequences. The year 1988 was a banner year for pulse sequence developments at NMRFAM. Use of ^1H detected ^1H - ^{15}N 2D correlation spectra of ^{15}N labeled proteins were introduced [115]. Stimulated by our ^{13}C direct detection efforts, Masatsune Kainosho and his group in Tokyo, Japan, produced 85% [U- ^{13}C]leucine streptomyces subtilisin inhibitor (SSI), which we used for the first through-backbone sequential assignments at NMRFAM [128]. This involved the correlation of direct detect ^{13}C double quantum correlation (INADEQUATE), ^1H detected ^1H - ^{13}C HSQC, and ^1H - ^{13}C HMBC experiments. Further use of this technology of ^{13}C detected experiments was also used to assign ^{13}C spin systems to amino acid type in flavodoxin [114]. Carbonyl-amide correlations were also obtained using a ^{13}C detected ^{13}C - ^{15}N 2D experiment on a [60% $^{13}\text{C}'$ methionine, 60% UL- ^{15}N]SSI sample from the Kainosho group [129]. We demonstrated the concerted application of 2D ^1H - ^{13}C and ^1H - ^{15}N spectroscopy for protein NMR assignments [116] and the power of ^{13}C - ^{13}C correlations for side-chain assignments [93].

In the late 1980s, we had an ongoing collaboration with Slobodan Macura to study the details of the cross relaxation and exchange effects in proteins. The understanding we gained

came from a number of new pulse sequences designed to manipulate cross relaxation [62,63]. Using the ROE in combination with the NOE, gave rise to the elimination of cross relaxation during exchange experiments and elimination of exchange mediated spin diffusion [32], which allowed correction of structural errors in protein structures that were due to internal motions. Elimination of spin diffusion using block decoupling was used to improve the accuracy of NMR derived structures [33]. At that time, it was apparent that the non-linear nature of cross relaxation had significant effects on the accuracy of the protein structure, if not corrected. Unfortunately, over the years, experiments have fallen victim to approximations and black-box software, and the non-linear behavior has been all but forgotten.

Correlation of chemical shifts with macromolecular structure

Although early studies of iron-sulfur proteins had identified, hyperfine-shifted signals [95], much of our early work on paramagnetic proteins focused on the diamagnetic spectral regions, those not significantly broadened through electron nuclear interactions. The pulse sequences used in these studies were the same ones used to study diamagnetic proteins. It wasn't until John's graduate student, T.-M Chan, began in 1983 to study hyperfine shifted resonances in a [2Fe-2S] ferredoxin that the NMR facility entered new direction [16]. The lab focused on the temperature dependence of the ^1H and ^{13}C hyperfine shifts in different oxidation states. The only difference between these paramagnetic and diamagnetic signals, apart from the large temperature dependence, was that the linewidths were significantly larger than scalar coupling constants, and so coherence transfer was not thought to be possible. The only pulse sequences that could be used were simple single-pulse experiments, and as a result, it was difficult to assign the signals.

A postdoc in the lab, Hong Cheng, succeeded in introducing ^2H and ^{13}C into selective positions of the cysteine residues of the vegetative ferredoxin from *Anabaena* 7120, which are bound to the iron atoms of the [2Fe-2S] cluster [17]. The cysteine ^1H signals appeared at ~100–140 ppm. A difference decoupling experiment showed an observable coupling interaction between the ^{13}C - ^1H in the cysteine sidechain signals. The results were remarkable in that the difference decoupling experiments were run at a recycle delay of 6 ms with 6 million acquisitions were collected. Furthermore, the narrowest line width was ~1200 Hz whereas the ^1H - ^{13}C coupling constant was ~140 Hz. Richard Ernst visited our facility a few years after winning the Nobel prize and commented that he would have predicted the experiment wouldn't work. Milo was both somewhat uneasy and at the same time elated by Ernst's comment. Since this experiment had worked, it inspired Milo to become further interested in these systems. However, experiments designed to correlate amide ^1H with the attached ^{15}N in high-spin Fe^{2+} or Fe^{3+} rubredoxin failed because the estimated ^1H linewidth was ~170,000 Hz based on the linewidth observed for a substituted ^2H . Since the ^1H - ^{15}N coupling is ~90 Hz, there was no hope of observing coherence transfer.

However, the large hyperfine chemical shifts of the ^1H and ^{15}N signals were intriguing, in that amino acid substitutions in *Clostridium pasteurianum* rubredoxin that altered the redox potential of the protein were accompanied by significant changes in the hyperfine shifts in the ^{15}N signals [56]. Even deuterium exchange of the amide ^1H showed shift differences of

10–20 ppm. Earlier, by using multiple magnetic field strength alignment to measure the magnetic susceptibility tensor, we had determined that these shifts were not due to through-space pseudocontact shifts [126]. Making assignments of these hyperfine signals to atoms in residues near to or part of the four cysteine residues that ligate the iron came down to amino acid specific labeling of a lot of samples including specific labeling by chemical synthesis [58]. A few years earlier, we had success in collaborating with Frank Weinhold on the use of quantum mechanics to calculate NMR parameters [25–28]. When Milo mentioned these large chemical shifts in the paramagnetic proteins to Frank, he suggested that we try some more calculations since there had been recent success with calculations of chemical shifts. After several months, Milo discovered that there had been a miscommunication: the chemical shift calculations that Frank had referred to were for diamagnetic systems rather than from the Fermi contact interactions between unpaired electrons and nuclei. When Milo presented his results at a Weinhold group meeting that showed $R^2 > 0.9$ agreement between theory and experiment, Frank commented that Milo was “very lucky”.

These successful results lead to a number of papers correlating the experimental hyperfine shifts of ^{15}N with theory. One paper even showed that the NMR spectrometer could be used as an extremely expensive potentiometer for measuring redox potentials of paramagnetic proteins based on the hyperfine shifts [57].

In our study of experimental NMR approaches to paramagnetic proteins, we discovered that certain correlation experiments would work with paramagnetically broadened ^{13}C signals. $^{13}\text{C}^\alpha$ [$^{13}\text{C}'$] constant-time COSY and $^{13}\text{C}'$ detected ^{15}N difference decoupling experiments, with the assistance of quantum mechanical calculations ($R^2 = 0.98$), allowed the assignment of hyperfine shifted carbon signals to specific amino acids. We were also able to assign hyperfine signals in both oxidation states by titrating with a reducing agent.

One very interesting observation was that eight of the hyperfine shifted ^{13}C signals were 7–11 bonds away from the paramagnetic iron. Fermi contact contributions are transmitted through bonds and fall off as the overlap of molecular orbitals that exponentially decay in amplitude [131]. There is negligible transmission across seven bonds. The only interpretation, reasonable or not, was that there must be physical overlap between C-H orbitals and the p orbitals of S, which also overlap with the orbitals of the paramagnetic Fe. This is a hydrogen-bond like interaction. Quantum mechanics, once again, verified that indeed a 3-center 4-electron bond exists between the CH antibonding orbitals and the bonding p -orbitals on S causing these large hyperfine shifts. The computed strength of these bonds was much weaker than a typical H-bond, but for a redox active protein, these interactions form a “wire” between the buried Fe and the surface of the protein allowing electron transfer to occur.

Research themes

One ongoing theme has been the use of NMR approaches to study conformational equilibria in several proteins, including staphylococcal nuclease [60,61,69], ribonuclease A [76], azurin [8], turkey ovomucoid third domain [112], and IscU [10,48]. A second theme was been the derivation of thermodynamic data on proteins from the pH-dependence of NMR

spectra [71,99,110,111,125]. A third theme has been NMR and quantum chemical investigations of H-bonding strengths, including H/D [59] and H/T [130] fractionation measurements, through H-bond couplings [2,133], and hyperfine chemical shift measurements [57,132]. And a final theme has been the development of software approaches to streamline and automate biomolecular NMR: this has included r-NMR for metabolomics [55] and the various software packages contained in servers accessible from the Integrative NMR package [54].

Our crystal ball to the future

Sample preparation likely will remain the major bottleneck for biomolecular NMR. We are past the era of low hanging fruit and into the more challenging era of intrinsically disordered proteins, membrane proteins, protein-nucleic acid complexes, and components of molecular machines. NMR spectroscopy will continue building on its strength for identifying conformational heterogeneity and excited states in biomacromolecules. Extrapolating from the past, we confidently predict that spectrometers operating at ^1H NMR frequencies of 1.2 GHz and above, will prove to be valuable for many biological applications because of their higher resolution, greater degree of first-order coupling, and a time scale that will enable the detection of some signals currently in the dark zone. The great expense of such spectrometers may also lead to the revival of large-scale NMR facilities. Another trend that is expected to grow is the application of NMR as a hybrid technique along with, X-ray, SAXS-WAXS [18], cryo-EM [30,49], or other modality. NMR spectroscopy and cryo-EM appear to be particularly complementary because they both do not require crystallization [20]. ^{19}F -labeling of proteins appears poised for a renaissance [109]. Solid state NMR with ultra-fast magic angle spinning [94] and signal enhancement by DNP [106] are expected to continue their rapid development. The seamless interoperability of biomolecular NMR software packages under a virtual machine [54] is likely to become more refined through the NMRbox project (<https://www.nmrbox.org/>).

Acknowledgments

We are indebted to our students, and postdocs as well as the many staff members of PUBMRL and NMRFAM (past and present) for their contributions. We thank Eldon L. Ulrich for critical comments on the manuscript and the National Institutes of Health for continuous support over 46 years. Current NIH support is from GM103399 (for NMRFAM) and GM109046 (for BMRB).

References

1. Allerhand A, Childers RF, Oldfield E. Natural-abundance carbon-13 nuclear magnetic resonance studies in 20-mm sample tubes. Observation of numerous single-carbon resonances of hen egg-white lysozyme. *Biochemistry*. 1973; 12:1335–1341. [PubMed: 4735301]
2. Assadi-Porter FM, Abildgaard F, Blad H, Markley JL. Correlation of the sweetness of variants of the protein brazzein with patterns of hydrogen bonds detected by NMR spectroscopy. *J Biol Chem*. 2003; 278:31331–31339. [PubMed: 12732626]
3. Bahrami A, Tonelli M, Sahu SC, Singarapu KK, Eghbalnia HR, Markley JL. Robust, integrated computational control of NMR experiments to achieve optimal assignment by ADAPT-NMR. *PLoS One*. 2012; 7:e33173. [PubMed: 22427982]

4. Baillargeon MW, Laskowski M Jr, Neves DE, Porubcan MA, Santini RE, Markley JL. Soybean trypsin inhibitor (kunitz) and its complex with trypsin. Carbon-13 nuclear magnetic resonance studies of the reactive site arginine. *Biochemistry*. 1980; 19:5703. [PubMed: 7459339]
5. Bax A. Two-dimensional Nuclear Magnetic Resonance in Liquids, Delft University Press, Delft. 1982
6. Beebe ET, Makino S, Markley JL, Fox BG. Automated cell-free protein production methods for structural studies. *Methods Mol Biol*. 2014; 1140:117–135. [PubMed: 24590713]
7. Berman H, Henrick K, Nakamura H, Markley JL. The worldwide Protein Data Bank (wwPDB): ensuring a single, uniform archive of PDB data. *Nucleic Acids Res*. 2007; 35:D301–D303. [PubMed: 17142228]
8. Blaszkak JA, Ulrich EL, Markley JL, McMillin DR. High-resolution proton nuclear magnetic resonance studies of the nickel(II) derivative of azurin. *Biochemistry*. 1982; 21:6253–6258. [PubMed: 6817786]
9. Bode W, Huber R. Crystal structure analysis and refinement of two variants of trigonal trypsinogen: trigonal trypsin and PEG (polyethylene glycol) trypsinogen and their comparison with orthorhombic trypsin and trigonal trypsinogen. *FEBS Lett*. 1978; 90:265–269. [PubMed: 668890]
10. Bothe JR, Tonelli M, Ali IK, Dai Z, Frederick RO, Westler WM, Markley JL. The complex energy landscape of the protein IscU. *Biophys J*. 2015; 109:1019–1025. [PubMed: 26331259]
11. Bothner-By AA, Dadok J. NMR spectroscopy at 600 MHz. *ACS Symp Ser*. 1982; 191:31–45. Chapter 3.
12. Browne DT, Kenyon GL, Packer EL, Sternlicht H, Wilson DM. Studies of macromolecular structure by ¹³C nuclear magnetic resonance. II. A specific labeling approach to the study of histidine residues in proteins. *J Am Chem Soc*. 1973; 95:1316–1323. [PubMed: 4569190]
13. Campbell ID, Dobson CM, Williams RJ, Wright PE. Pulse methods for the simplification of protein NMR spectra. *FEBS Lett*. 1975; 57:96–99. [PubMed: 1175783]
14. Chaiken IM, Freedman MH, Lyster JR Jr, Cohen JS. Preparation and studies of ¹⁹F-labeled and enriched ¹³C-labeled semisynthetic ribonuclease-S' analogues. *J Biol Chem*. 1973; 248:884–891. [PubMed: 4684711]
15. Chan TM, Ulrich EL, Markley JL. Balaban International Science Services, Philadelphia, PA. 1980:697–704.
16. Chan TM, Markley JL. Nuclear magnetic resonance studies of two-iron-two-sulfur ferredoxins. 5. Hyperfine-shifted peaks in Hydrogen-1 and Carbon-13 spectra. *Biochemistry*. 1983; 22:6008–6010.
17. Cheng H, Westler WM, Xia B, Oh BH, Markley JL. Protein expression, selective isotopic labeling, and analysis of hyperfine-shifted NMR signals of *Anabaena* 7120 vegetative [2Fe-2S] ferredoxin. *Arch Biochem Biophys*. 1995; 316:619–634. [PubMed: 7840674]
18. Cornilescu G, Didychuk AL, Rodgers ML, Michael LA, Burke JE, Montemayor EJ, Hoskins AA, Butcher SE. Structural Analysis of Multi-Helical RNAs by NMR-SAXS/WAXS: application to the U4/U6 di-snRNA. *J Mol Biol*. 2016; 428:777–789. [PubMed: 26655855]
19. Crespi HL, Katz JJ. Preparation of deuterated proteins and enzymes. *Methods Enzymol*. 1972; 26:627–637. [PubMed: 4376580]
20. Cuniasse P, Tavares P, Orlova EV, Zinn-Justin S. Structures of biomolecular complexes by combination of NMR and cryoEM methods. *Curr Opin Struct Biol*. 2017; 43:104–113. [PubMed: 28056362]
21. Cusumano CL, Taniuchi H, Anfinsen CB. Staphylococcal nuclease (Foggi strain). I. Order of cyanogen bromide fragments and a “fourth” histidine residue. *J Biol Chem*. 1968; 243:4769–4777. [PubMed: 5687719]
22. Dadok J, Sprecher RF. Correlation NMR spectroscopy. *J Magn Reson*. 1974; 13:243–248.
23. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPIPE - a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR*. 1995; 6:277–293. [PubMed: 8520220]
24. Eakin RT, Morgan LO, Gregg CT, Matwiyoff NA. Carbon-13 nuclear magnetic resonance spectroscopy of living cells and their metabolism of a specifically labeled ¹³C substrate. *FEBS Lett*. 1972; 28:259–264. [PubMed: 11946872]

25. Edison AS, Markley JL, Weinhold F. Calculation of nuclear spin-spin coupling constants with ab initio molecular orbital wave functions. *J Phys Chem.* 1993; 97:11657–11665.
26. Edison AS, Markley JL, Weinhold F. Calculations of one-, two- and three-bond nuclear spin-spin couplings in a model peptide and correlations with experimental data. *J Biomol NMR.* 1994; 4:519–542. [PubMed: 8075539]
27. Edison AS, Markley JL, Weinhold F. Ab initio calculations of protium deuterium fractionation factors in O 2 H 5pclusters. *J Phys Chem.* 1995; 99:8013–8016.
28. Edison AS, Weinhold F, Markley JL. Theoretical studies of protium/deuterium fractionation factors and cooperative hydrogen bonding in peptides. *J Am Chem Soc.* 1995; 117:9619–9624.
29. Ehrenberg, A. Malmström, BG., Vänngård, T., editors. Proceedings of the Second International Conference Held at the Wennergren Center, Stockholm, June, 1966. Pergamon Press; London: 1967. Magnetic Resonance in Biological Systems.
30. Eichmann C, Campioni S, Kowal J, Maslennikov I, Gerez J, Liu X, Verasdonck J, Nespovitaya N, Choe S, Meier BH, Picotti P, Rizo J, Stahlberg H, Riek R. Preparation and characterization of stable alpha-synuclein lipoprotein particles. *J Biol Chem.* 2016; 291:8516–8527. [PubMed: 26846854]
31. Ernst RR, Anderson WA. Application of Fourier transform spectroscopy to magnetic resonance. *Rev Sci Instrum.* 1966; 37:93–102.
32. Fejzo J, Westler WM, Macura S, Markley JL. Elimination of cross-relaxation effects from two-dimensional chemical-exchange spectra of macromolecules. *J Am Chem Soc.* 1990; 112:2574–2577.
33. Fejzo J, Krezel AM, Westler WM, Macura S, Markley JL. Refinement of the NMR solution structure of a protein to remove distortions arising from neglect of internal motion. *Biochemistry.* 1991; 30:3807–3811. [PubMed: 1850288]
34. Frederick RO, Bergeman L, Blommel PG, Bailey LJ, McCoy JG, Song J, Meske L, Bingman CA, Ritters M, Dillon NA, Kunert J, Yoon JW, Lim A, Cassidy M, Bunge J, Aceti DJ, Primm JG, Markley JL, Phillips GN Jr, Fox BG. Small-scale, semi-automated purification of eukaryotic proteins for structure determination. *J Struct Funct Genomics.* 2007; 8:153–166. [PubMed: 17985212]
35. Gutmanas A, Adams PD, Bardiaux B, Berman HM, Case DA, Fogh RH, Guntert P, Hendrickx PM, Herrmann T, Kleywegt GJ, Kobayashi N, Lange OF, Markley JL, Montelione GT, Nilges M, Ragan TJ, Schwieters CD, Tejero R, Ulrich EL, Velankar S, Vranken WF, Wedell JR, Westbrook J, Wishart DS, Vuister GW. NMR Exchange Format: a unified and open standard for representation of NMR restraint data. *Nat Struct Mol Biol.* 2015; 22:433–434. [PubMed: 26036565]
36. Hahn EL. Spin echoes. *Phys Rev.* 1950; 80:580–594.
37. Hahn EL, Maxwell DE. Spin echo measurements of nuclear spin coupling in molecules. *Phys Rev.* 1952; 88:1070–1084.
38. Hall SR. The STAR file: a new format for electronic data transfer and archiving. *J Chem Inf Comput Sci.* 1991; 31:326–333.
39. Hall SR, Allen FH, Brown ID. International union of crystallography, commission on crystallographic data, commission on journals, working party on crystallographic information, the crystallographic information file (CIF): a new standard archive file for crystallography. *Acta Crystallogr A.* 1991; 47:655–685.
40. Hall SR, Cook APF. STAR dictionary definition language: initial specification. *J Chem Inf Comput Sci.* 1995; 35:819–825.
41. Horsley W, Sternlicht H, Cohen JS. Carbon-13 magnetic resonance studies of carbon-13 enriched amino acids. *Biochem Biophys Res Commun.* 1969; 37:47–51. [PubMed: 5346360]
42. Jardetzky CD, Jardetzky O. Investigation of the structure of purines, pyrimidines, ribose nucleosides and nucleotides by proton magnetic resonance. *J Am Chem Soc.* 1960; 82:222–229.
43. Jardetzky O, Jardetzky CD. An interpretation of the proton magnetic resonance spectrum of ribonuclease. *J Am Chem Soc.* 1957; 79:5322–5323.
44. Jardetzky O, Jardetzky CD. Proton magnetic resonance spectra of amino acids. *J Biol Chem.* 1958; 233:383–387. [PubMed: 13563506]

45. Jardetzky O, Wade NJ, Fisher JJ. Proton magnetic resonance investigations of enzyme-coenzyme complexes. *Nature*. 1963; 197:183–184. [PubMed: 13957483]
46. Jardetzky O. Proceedings of the International Symposium on Nuclear Magnetic Resonance. 1965:N-3–N-14. Tokyo, Japan.
47. Kay LE, Ikura M, Tschudin R, Bax A. 3-Dimensional triple-resonance NMR-spectroscopy of isotopically enriched proteins. *J Magn Reson*. 1990; 89:496–514.
48. Kim JH, Füzery AK, Tonelli M, Ta DT, Westler WM, Vickery LE, Markley JL. Structure and dynamics of the iron-sulfur cluster assembly scaffold protein IscU and its interaction with the cochaperone HscB. *Biochemistry*. 2009; 48:6062–6071. [PubMed: 19492851]
49. Knoops K, Schoehn G, Schaffitzel C. Cryo-electron microscopy of ribosomal complexes in cotranslational folding, targeting, and translocation. *Wiley Interdiscip Rev RNA*. 2012; 3:429–441. [PubMed: 22095783]
50. Kojiro CL, Markley JL. Connectivity of proton and carbon spectra of plastocyanin established by two-dimensional nuclear magnetic resonance. *FEBS Lett*. 1983; 162:52–56.
51. Kowalsky A. Nuclear magnetic resonance studies of proteins. *J Biol Chem*. 1962; 237:1807–1819. [PubMed: 14036907]
52. Kupce E, Freeman R. Reconstruction of the three-dimensional NMR spectrum of a protein from a set of plane projections. *J Biomol NMR*. 2003; 27:383–387. [PubMed: 14512735]
53. Lee W, Tonelli M, Markley JL. NMRFAM-SPARKY: enhanced software for biomolecular NMR spectroscopy. *Bioinformatics*. 2015; 31:1325–1327. [PubMed: 25505092]
54. Lee W, Cornilescu G, Dashti H, Eghbalian HR, Tonelli M, Westler WM, Butcher SE, Henzler-Wildman KA, Markley JL. Integrative NMR for biomolecular research. *J Biomol NMR*. 2016; 64:307–332. [PubMed: 27023095]
55. Lewis IA, Schommer SC, Markley JL. rNMR: open source software for identifying and quantifying metabolites in NMR spectra. *Magn Reson Chem*. 2009; 47:s123–s126. [PubMed: 19821464]
56. Lin IJ, Gebel EB, Machonkin TE, Westler WM, Markley JL. Correlation between hydrogen bond lengths and reduction potentials in *Clostridium pasteurianum* rubredoxin. *J Am Chem Soc*. 2003; 125:1464–1465. [PubMed: 12568591]
57. Lin IJ, Gebel EB, Machonkin TE, Westler WM, Markley JL. Changes in hydrogen-bond strengths explain reduction potentials in 10 rubredoxin variants. *Proc Natl Acad Sci U S A*. 2005; 102:14581–14586. [PubMed: 16199518]
58. Lin IJ, Xia B, King DS, Machonkin TE, Westler WM, Markley JL. Hyperfine-shifted ¹³C and ¹⁵N NMR signals from *Clostridium pasteurianum* rubredoxin: extensive assignments and quantum chemical verification. *J Am Chem Soc*. 2009; 131:15555–15563. [PubMed: 19799419]
59. Loh, SN., Markley, JL., Angeletti, R. Techniques in Protein Chemistry IV. Academic Press; San Diego: 1993. Measurement of Amide Hydrogen D/H Fractionation Factors in Proteins by NMR Spectroscopy; p. 517-524.
60. Loh SN, Prehoda KE, Wang J, Markley JL. Hydrogen exchange in unligated and ligated staphylococcal nuclease. *Biochemistry*. 1993; 32:11022–11028. [PubMed: 8218167]
61. Loh, SN., Prehoda, KE., Wang, J., Markley, JL., Crabb, JW. Techniques in Protein Chemistry V. Academic Press, Inc.; New York, NY: 1994. Measuring Global and Local Structural Free Energy Changes in Staphylococcal Nuclease by NMR-observed Hydrogen Exchange; p. 431-438.
62. Macura S, Fejzo J, Westler WM, Markley JL. Influence of slow internal motion in proteins on cross-relaxation rates determined by two-dimensional exchange spectroscopy. *Bull Magnetic Reson*. 1993; 16:69–87.
63. Macura S, Westler WM, Markley JL. Two-dimensional exchange spectroscopy of proteins. *Methods Enzymol*. 1994; 239:106–144. [PubMed: 7830582]
64. Makino S, Beebe ET, Markley JL, Fox BG. Cell-free protein synthesis for functional and structural studies. *Methods Mol Biol*. 2014; 1091:161–178. [PubMed: 24203331]
65. Mandel M. Proton magnetic resonance spectra of some proteins. I. Ribonuclease, oxidized ribonuclease, lysozyme, and cytochrome C. *J Biol Chem*. 1965; 240:1586–1592. [PubMed: 14285495]

66. Markley JL, Meadows DH, Jardetzky O. Nuclear magnetic resonance studies of helix-coil transitions in polyamino acids. *J Mol Biol.* 1967; 27:25–40. [PubMed: 6033611]
67. Markley JL, Putter I, Jardetzky O. High-resolution nuclear magnetic resonance spectra of selectively deuterated staphylococcal nuclease. *Science.* 1968; 161:1249–1251. [PubMed: 5673435]
68. Markley, JL. Proton Magnetic Resonance Studies of the Structure and Binding Sites of Staphylococcal Nuclease. Committee on Higher Degrees in Biophysics, Harvard University; Cambridge, Massachusetts: 1969. p. 271
69. Markley JL, Williams MN, Jardetzky O. Nuclear magnetic resonance studies of the structure and binding sites of enzymes. XII. A conformational equilibrium in staphylococcal nuclease involving a histidine residue. *Proc Natl Acad Sci U S A.* 1970; 65:645–651. [PubMed: 5267145]
70. Markley JL, Horsley WJ, Klein MP. Spin-lattice relaxation measurements in slowly relaxing complex spectra. *J Chem Phys.* 1971; 55:3604–3605.
71. Markley JL. Nuclear magnetic resonance studies of trypsin inhibitors. Histidines of virgin and modified soybean trypsin inhibitor (kunitz). *Biochemistry.* 1973; 12:2245–2250. [PubMed: 4710581]
72. Markley JL. High-resolution proton magnetic resonance studies of two trypsin inhibitors: soybean trypsin inhibitor (Kunitz) and ovomucoid (hen egg white). *Ann N Y Acad Sci.* 1973; 222:347–373. [PubMed: 4522435]
73. Markley JL, Cheung SM. Differential exchange of C2-hydrogens of histidine side chains in native proteins: proposed general technique for the assignment of histidine NMR peaks in proteins, in: *Proc Int Conf on Stable Isotopes in Chem, Biol and Med*, U S Atomic Energy Commission, Argonne, IL. 1973:103–118.
74. Markley JL. Correlation proton magnetic resonance studies at 250 MHz of bovine pancreatic ribonuclease. I. Reinvestigation of the histidine peak assignments. *Biochemistry.* 1975; 14:3546–3554. [PubMed: 240382]
75. Markley JL. Observation of histidine residues in proteins by means of nuclear magnetic resonance spectroscopy. *Acc Chem Res.* 1975; 8:70–80.
76. Markley JL. Correlation proton magnetic resonance studies at 250 MHz of bovine pancreatic ribonuclease. II. pH and inhibitor-induced conformational transitions affecting histidine-48 and one tyrosine residue of ribonuclease A. *Biochemistry.* 1975; 14:554–561.
77. Markley JL, Finkenstadt WR. Correlation proton magnetic resonance studies at 250 MHz of bovine pancreatic ribonuclease. III. Mutual electrostatic interaction between histidine residues 12 and 119. *Biochemistry.* 1975; 14:3562–3566. [PubMed: 240383]
78. Markley JL, Kato I. Assignment of the histidine proton magnetic resonance peaks of soybean trypsin inhibitor (Kunitz) by a differential deuterium exchange technique. *Biochemistry.* 1975; 14:3234–3237. [PubMed: 238587]
79. Markley JL, Ulrich EL, Berg SP, Krogmann DW. Nuclear magnetic resonance studies of the copper binding sites of blue copper proteins: oxidized, reduced, and apoplastocyanin. *Biochemistry.* 1975; 14:4428–4433. [PubMed: 809054]
80. Markley JL, Porubcan MA. The charge-relay system of serine proteinases: proton magnetic resonance titration studies of the four histidines of porcine trypsin. *J Mol Biol.* 1976; 102:487–509. [PubMed: 5610]
81. Markley JL, Ulrich EL, Krogmann DW. Spinach plastocyanin: comparison of reduced and oxidized forms by natural abundance carbon-13 nuclear magnetic resonance spectroscopy. *Biochem Biophys Res Commun.* 1977; 78:106–114. [PubMed: 907662]
82. Markley JL. Hydrogen bonds in serine proteinases and their complexes with protein proteinase inhibitors. Proton nuclear magnetic resonance studies. *Biochemistry.* 1978; 17:4648–4656. [PubMed: 569493]
83. Markley JL, Ibanez IB. Zymogen activation in serine proteinases. Proton magnetic resonance pH titration studies of the two histidines of bovine chymotrypsinogen A and chymotrypsin Aalpha. *Biochemistry.* 1978; 17:4627–4640. [PubMed: 31898]
84. Markley JL, Neves DE, Westler WM, Ibanez IB, Porubcan MA, Baillargeon MW. NMR studies of serine proteinases. *Dev Biochem.* 1980; 10:31–62.

85. Markley JL, Ulrich EL. Detailed analysis of protein structure and function by NMR spectroscopy: survey of resonance assignments. *Annu Rev Biophys Bioeng.* 1984; 13:493–521. [PubMed: 6331288]
86. Markley JL, Westler WM, Chan TM, Kojiro CL, Ulrich EL. Two-dimensional NMR approaches to the study of protein structure and function. *Fed Proc.* 1984; 43:2648–2656. [PubMed: 6430721]
87. Markley JL, Bax A, Arata Y, Hilbers CW, Kaptein R, Sykes BD, Wright PE, Wüthrich K. Recommendations for the presentation of NMR structures of proteins and nucleic acids (IUPAC recommendations 1998). *Pure Appl Chem.* 1998; 70:117–142.
88. Markley JL, Aceti DJ, Bingman CA, Fox BG, Frederick RO, Makino S, Nichols KW, Phillips GN Jr, Primm JG, Sahu SC, Vojtik FC, Volkman BF, Wrobel RL, Zolnai Z. The center for eukaryotic structural genomics. *J Struct Funct Genomics.* 2009; 10:165–179. [PubMed: 19130299]
89. Markley JL, Akutsu H, Asakura T, Baldus M, Boelens R, Bonvin A, Kaptein R, Bax A, Bezsonova I, Gryk MR, Hoch JC, Korzhnev DM, Maciejewski MW, Case D, Chazin WJ, Cross TA, Dames S, Kessler H, Lange O, Madl T, Reif B, Sattler M, Eliezer D, Fersht A, Forman-Kay J, Kay LE, Fraser J, Gross J, Kortemme T, Sali A, Fujiwara T, Gardner K, Luo X, Rizo-Rey J, Rosen M, Gil RR, Ho C, Rule G, Gronenborn AM, Ishima R, Klein-Seetharaman J, Tang P, van der Wel P, Xu Y, Grzesiek S, Hiller S, Seelig J, Laue ED, Mott H, Nietlispach D, Barsukov I, Lian LY, Middleton D, Blumenschein T, Moore G, Campbell I, Schnell J, Vakonakis IJ, Watts A, Conte MR, Mason J, Pfuhl M, Sanderson MR, Craven J, Williamson M, Dominguez C, Roberts G, Gunther U, Overduin M, Werner J, Williamson P, Blindauer C, Crump M, Driscoll P, Frenkiel T, Golovanov A, Matthews S, Parkinson J, Uhrin D, Williams M, Neuhaus D, Oschkinat H, Ramos A, Shaw DE, Steinbeck C, Vendruscolo M, Vuister GW, Walters KJ, Weinstein H, Wuthrich K, Yokoyama S. In support of the BMRB. *Nat Struct Mol Biol.* 2012; 19:854–860. [PubMed: 22955930]
90. McDonald, CC., Phillips, WD. *Magnetic Resonance in Biological Systems*, Proceedings of the Second International Conference Held at the Wenner-gren Center, Stockholm, June 1966. Pergamon Press; London: 1967. NMR studies of biological macromolecules; p. 428
91. Meadows DH, Markley JL, Cohen JS, Jardetzky O. Nuclear magnetic resonance studies of the structure and binding sites of enzymes. I. Histidine residues. *Proc Natl Acad Sci U S A.* 1967; 58:1307–1313. [PubMed: 5237865]
92. Morris GA, Freeman R. Enhancement of nuclear magnetic resonance signals by polarization transfer. *J Am Chem Soc.* 1979; 101:760–762.
93. Oh BH, Westler WM, Darba P, Markley JL. Protein carbon-13 spin systems by a single two-dimensional nuclear magnetic resonance experiment. *Science.* 1988; 240:908–911. [PubMed: 3129784]
94. Pandey MK, Zhang R, Hashi K, Ohki S, Nishijima G, Matsumoto S, Noguchi T, Deguchi K, Goto A, Shimizu T, Maeda H, Takahashi M, Yanagisawa Y, Yamazaki T, Iguchi S, Tanaka R, Nemoto T, Miyamoto T, Suematsu H, Saito K, Miki T, Ramamoorthy A, Nishiyama Y. 1020MHz single-channel proton fast magic angle spinning solid-state NMR spectroscopy. *J Magn Reson.* 2015; 261:1–5. [PubMed: 26524647]
95. Poe M, Phillips WD, McDonald CC, Lovenberg W. Proton magnetic resonance study of ferredoxin from *Clostridium pasteurianum*. *Proc Natl Acad Sci U S A.* 1970; 65:797–804. [PubMed: 5266908]
96. Porubcan MA, Neves DE, Rausch SK, Markley JL. Active site in zymogens. Proton magnetic resonance pH titration curves of histidine-57 in porcine and bovine trypsinogens and in their complexes with bovine pancreatic trypsin inhibitor (Kunitz). *Biochemistry.* 1978; 17:4640–4647. [PubMed: 31899]
97. Porubcan MA, Westler WM, Ibanez IB, Markley JL. (Diisopropylphosphoryl)serine proteinases. Proton and phosphorus-31 nuclear magnetic resonance-pH titration studies. *Biochemistry.* 1979; 18:4108–4116. [PubMed: 39591]
98. Putter I, Barreto A, Markley JL, Jardetzky O. Nuclear magnetic resonance studies of the structure and binding sites of enzymes. X. Preparation of selectively deuterated analogs of staphylococcal nuclease. *Proc Natl Acad Sci U S A.* 1969; 64:1396–1403. [PubMed: 5271760]
99. Qasim MA, Ranjbar MR, Wynn R, Anderson S, Laskowski M Jr. Ionizable P1 residues in serine proteinase inhibitors undergo large pK shifts on complex formation. *J Biol Chem.* 1995; 270:27419–27422. [PubMed: 7499197]

100. Redfield AG, Kunz SD, Ralph EK. Dyn. range Fourier transform Prot. magnetic Reson. J Magn Reson. 1975; 19:114–117.
101. Richarz R, Tschesche H, Wuthrich K. Carbon-13 nuclear magnetic resonance studies of the selectively isotope-labeled reactive site peptide bond of the basic pancreatic trypsin inhibitor in the complexes with trypsin, trypsinogen, and anhydrotrypsin. Biochemistry. 1980; 19:5711–5715. [PubMed: 6161642]
102. Robillard G, Shulman RG. High resolution nuclear magnetic resonance study of the histidine-aspartate hydrogen bond in chymotrypsin and chymotrypsinogen. J Mol Biol. 1972; 71:507–511. [PubMed: 4635995]
103. Robillard G, Shulman RG. High resolution NMR study of the charge relay system in chymotrypsin. Ann N Y Acad Sci. 1973; 222:220–225. [PubMed: 4522429]
104. Robillard G, Shaw E, Shulman RG. ¹³C high-resolution nuclear magnetic resonance studies of enzyme-substrate reactions at equilibrium. Substrate studies of chymotrypsin-N-acetyltyrosine semicarbazide complexes. Proc Natl Acad Sci U S A. 1974; 71:2623–2626. [PubMed: 4528121]
105. Robillard G, Shulman RG. High resolution nuclear magnetic resonance studies of the active site of chymotrypsin. II. Polarization of histidine 57 by substrate analogues and competitive inhibitors. J Mol Biol. 1974; 86:541–558. [PubMed: 4852270]
106. Rosay M, Blank M, Engelke F. Instrumentation for solid-state dynamic nuclear polarization with magic angle spinning NMR. J Magn Reson. 2016; 264:88–98. [PubMed: 26920834]
107. Saunders M, Wishnia A, Kirkwood JG. The nuclear magnetic resonance spectrum of ribonuclease. J Am Chem Soc. 1957; 79:3289–3290.
108. Sealock RW, Laskowski M Jr. Enzymatic replacement of the arginyl by a lysyl residue in the reactive site of soybean trypsin inhibitor. Biochemistry. 1969; 8:3703–3710. [PubMed: 5387527]
109. Sharaf NG, Gronenborn AM. (19)F-modified proteins and (19)F-containing ligands as tools in solution NMR studies of protein interactions. Methods Enzymol. 2015; 565:67–95. [PubMed: 26577728]
110. Song J, Markley JL. NMR chemical shift mapping of the binding site of a protein proteinase inhibitor: changes in the (1)H, (13)C and (15)N NMR chemical shifts of Turkey ovomucoid third domain upon binding to bovine chymotrypsin A(alpha). J MolRecognit. 2001; 14:166–171.
111. Song J, Laskowski M Jr, Qasim MA, Markley JL. NMR determination of pKa values for Asp, Glu, His, and Lys mutants at each variable contiguous enzyme-inhibitor contact position of the Turkey ovomucoid third domain. Biochemistry. 2003; 42:2847–2856. [PubMed: 12627950]
112. Song J, Laskowski M Jr, Qasim MA, Markley JL. Two conformational states of Turkey ovomucoid third domain at low pH: three-dimensional structures, internal dynamics, and interconversion kinetics and thermodynamics. Biochemistry. 2003; 42:6380–6391. [PubMed: 12767219]
113. Sørensen OW, Eich GW, Levitt MH, Bodenhausen G, Ernst RR. Product operator formalism for the description of NMR pulse experiments. Prog NMR Spectrosc. 1983; 16:163–192.
114. Stockman BJ, Westler WM, Darba P, Markley JL. Detailed analysis of Carbon-13 NMR spin systems in a uniformly Carbon-13 enriched protein: flavodoxin from *Anabaena* 7120. J Am Chem Soc. 1988; 110:4096–4097.
115. Stockman BJ, Westler WM, Mooberry ES, Markley JL. Flavodoxin from *Anabaena* 7120: uniform nitrogen-15 enrichment and hydrogen-1, nitrogen-15, and phosphorus-31 NMR investigations of the flavin mononucleotide binding site in the reduced and oxidized states. Biochemistry. 1988; 27:136–142. [PubMed: 3126808]
116. Stockman BJ, Reily MD, Westler WM, Ulrich EL, Markley JL. Concerted two-dimensional NMR approaches to hydrogen-1, carbon-13, and nitrogen-15 resonance assignments in proteins. Biochemistry. 1989; 28:230–236. [PubMed: 2539856]
117. Sweet RM, Wright HT, Janin J, Chothia CH, Blow DM. Crystal structure of the complex of porcine trypsin with soybean trypsin inhibitor (kunitz) at 2.6 resolution. Biochemistry. 1974; 13:4212–4228. [PubMed: 4472048]
118. Taniuchi H, Anfinsen CB, Sodja A. The amino acid sequence of an extracellular nuclease of *Staphylococcus aureus*. 3. Complete amino acid sequence. J Biol Chem. 1967; 242:4752–4758. [PubMed: 4294024]

119. Tyler RC, Aceti DJ, Bingman CA, Cornilescu CC, Fox BG, Frederick RO, Jeon WB, Lee MS, Newman CS, Peterson FC, Phillips GN Jr, Shahan MN, Singh S, Song J, Sreenath HK, Tyler EM, Ulrich EL, Vinarov DA, Vojtik FC, Volkman BF, Wrobel RL, Zhao Q, Markley JL. Comparison of cell-based and cell-free protocols for producing target proteins from the *Arabidopsis thaliana* genome for structural studies. *Proteins*. 2005; 59:633–643. [PubMed: 15789406]
120. Ulrich EL, Markley JL, Kyogoku Y. Creation of a nuclear magnetic resonance data repository and literature database. *Protein Seq Data Anal*. 1989; 2:23–37. [PubMed: 2911559]
121. Ulrich EL, Argentar D, Klimowicz A, Markley JL. STAR/CIF macromolecular NMR data dictionaries and data file formats. *Acta Crystallogr A*. 1996; 52(a1):C577–C577.
122. Vinarov DA, Lytle BL, Peterson FC, Tyler EM, Volkman BF, Markley JL. Cell-free protein production and labeling protocol for NMR-based structural proteomics. *Nat Methods*. 2004; 1:149–153. [PubMed: 15782178]
123. Vinarov DA, Markley JL. High-throughput automated platform for nuclear magnetic resonance-based structural proteomics. *Expert Rev Proteomics*. 2005; 2:49–55. [PubMed: 15966852]
124. Vinarov DA, Loushin Newman CL, Tyler EM, Markley JL. Protein production using the wheat germ cell-free expression system. *Curr Protoc Protein Sci*. 2006; 5.18.11–15.18.18.
125. Virudachalam R, Low PS, Argos P, Markley JL. Turnip yellow mosaic virus and its capsid have thermal stabilities with opposite pH dependence: studies by differential scanning calorimetry and ³¹P nuclear magnetic resonance spectroscopy. *Virology*. 1985; 146:213–220. [PubMed: 4049734]
126. Volkman BF, Wilkens SJ, Lee AL, Xia B, Westler WM, Beger R, Markley JL. Redox-dependent magnetic alignment of *Clostridium pasteurianum* rubredoxin: measurement of magnetic susceptibility anisotropy and prediction of pseudocontact shift contributions. *J Am Chem Soc*. 1999; 121:4677–4683.
127. Westler WM, Markley JL, Bachovchin WW. Proton NMR spectroscopy of the active site histidine of alpha-lytic proteinase. Effects of adjacent ¹³C and ¹⁵N labels. *FEBS Lett*. 1982; 138:233–235. [PubMed: 7040114]
128. Westler WM, Kainosho M, Nagao H, Tomonaga N, Markley JL. Two-dimensional NMR strategies for carbon-carbon correlations and sequence-specific assignments in Carbon-13 labeled proteins. *J Am Chem Soc*. 1988; 110:4093–4095.
129. Westler WM, Stockman BJ, Hosoya Y, Miyake Y, Kainosho M, Markley JL. Correlation of Carbon-13 and Nitrogen-15 chemical shifts in uniformly labeled proteins by heteronuclear two-dimensional NMR spectroscopy. *J Am Chem Soc*. 1988; 110:6265–6258. [PubMed: 22148822]
130. Westler WM, Frey PA, Lin J, Wemmer DE, Morimoto H, Williams PG, Markley JL. Evidence for a strong hydrogen bond in the catalytic dyad of transition-state analogue inhibitor complexes of chymotrypsin from proton-triton NMR isotope shifts. *J Am Chem Soc*. 2002; 124:4196–4197. [PubMed: 11960433]
131. Westler WM, Lin JJ, Perczel A, Weinhold F, Markley JL. Hyperfine-shifted (¹³C) resonance assignments in an iron-sulfur protein with quantum chemical verification: aliphatic C-H center dot center dot center dot S 3-center-4-electron interactions. *J Am Chem Soc*. 2011; 133:1310–1316. [PubMed: 21207994]
132. Wilkens SJ, Xia B, Weinhold F, Markley JL, Westler WM. NMR investigations of *Clostridium pasteurianum* rubredoxin - origin of hyperfine ¹H, ²H, ¹³C, and ¹⁵N NMR chemical shifts in iron-sulfur proteins as determined by comparison of experimental data with hybrid density functional calculations. *J Am Chem Soc*. 1998; 120:4806–4814.
133. Wilkens SJ, Westler WM, Weinhold F, Markley JL. Trans-hydrogen-bond h2JNN and h1JNH couplings in the DNA a-t base pair: natural bond orbital analysis. *J Am Chem Soc*. 2002; 124:1190–1191. [PubMed: 11841286]
134. Wüthrich K. *NMR in Biological Research: Peptides and Proteins*, North-Holland Publishing Company. 1976
135. Wüthrich, K. *NMR of Proteins and Nucleic Acids*. Wiley Interscience; New York, NY: 1986.
136. Zehfus MH, Reily MD, Ulrich EL, Westler WM, Markley JL. ¹H, ¹³C, and ¹⁵N resonance assignments for a ferrocycytochrome c 553 heme by multinuclear NMR spectroscopy. *Arch Biochem Biophys*. 1990; 276:369–373. [PubMed: 2154947]

Fig. 1. Biomolecular NMR group at the Merck Institute, Rahway, New Jersey, in 1967. From left to right: Donella Meadows, Oleg Jardetzky, Jack Cohen, and John Markley. Jack Cohen had a long research career at the NIH and later in Israel. Donella Meadows left NMR after her PhD to become a pioneering environmental scientist, teacher, and writer. She is best known as the lead author of *The Limits to Growth* and *Thinking in Systems: a Primer*. She was a Pew Scholar in Conservation and Environment and a MacArthur Fellow. Photograph from Business Week, December 2, 1967, page 94; reprinted with permission.

Fig. 2. Varian HR-220 (220 MHz) NMR spectrometer and console, Calcomp 565 chart recorder, home-built pulse gear, PDP-11 computer, and magnetic tape recorder in Mel Klein's laboratory at UC Berkeley. Bill Horsely, who worked with John Markley, is sitting at the NMR spectrometer. Reproduced from "Chemical Biodynamics Group," Lawrence Radiation Laboratory, University of California, Berkeley, California, USAEC Pub. No. 33, September 1970, p. 6.

Fig. 3.
Milo Westler and Nicolet NT-1180 computer and console at PUBMRL in 1979. Photo taken by JLM.

Fig. 4.
Brian Stockman observing a quench during the installation of the Bruker 600 at NMRFAM in 1988. Photo taken by JLM.

Fig. 5. Delivery of the Varian 900 MHz NMR spectrometer at NMRFAM in 2003. The facility was designed with a hatch in the ceiling to accommodate large pieces of equipment. Photo taken by JLM.

Fig. 6.
NMRFAM spectrometers (plot of field strength vs. delivery date).

Fig. 7.
Array processor used for early processing of 3D NMR data at NMRFAM. Photo taken by WMW.

Fig. 8.

High frequency region of 100 MHz ¹H NMR spectra of (a) unlabeled staphylococcal nuclease and (b) selectively-deuterated staphylococcal nuclease. From publication [98]. Tryptophan was added to the growth medium containing deuterated amino acids. Signals from histidine and tyrosine were present because of back exchange during HCl hydrolysis of the deuterated algal protein that was the source of the deuterated amino acids. Reprinted from Fig. 1, Proc. Natl. Acad. Sci. U.S.A. 64:1399 (1969).

Fig. 9.

Comparison of the 100 MHz NMR spectra of the histidine ring proton regions of staphylococcal nuclease (Nase) isolated from the Foggi (a) and (c) and V8 (b) and (d) strains of *Staphylococcus aureus* [68]. Because amino acid sequencing showed that residue 174 in Nase from the Foggi strain is histidine whereas it is leucine in Nase from the V8 strain [21], the spectra enable peak H3 to be assigned to H124. Reprinted from John Markley's Ph.D. thesis, p. 165.