ML-01 Fifty Years of NMR in Japan

Yoji Arata

Important contributions of Japanese scientists to the advancement of theoretical and experimental aspects of NMR in the past fifty years will be briefly described.

ML-02 Characterization of transient protein folding and unfolding processes by NMR relaxation dispersion

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NMR relaxation measurements provide a powerful approach for direct experimental characterization of protein dynamics and protein folding processes on a broad range of time scales, ranging from ps to ms, and yield unique insights into the protein energy landscape. In particular, relaxation dispersion experiments permit quantitative analysis of the kinetics and mechanism of spontaneous protein folding and unfolding events under equilibrium conditions. Dispersion experiments also provide chemical shift data that allow detailed structural characterization of weakly populated folding intermediates. Applications of NMR to study kinetic folding and unfolding pathways of apomyoglobin will be discussed. Native apomyoglobin unfolds on a sequential pathway via two intermediates: an intermediate that involves local unfolding of one helix, and a disordered molten globule intermediate. Analysis of transient state chemical shifts reveals the location and population of residual helical structure in the intermediates and identifies regions that unfold or rearrange into non-native structure during the transition to the molten globule state. The experiments also identify regions of energetic frustration that “crack” during unfolding and impede the refolding process. Relaxation dispersion measurements on acid-denatured states of apomyoglobin provide novel insights into the earliest steps in the refolding process. Folding is seen to proceed along a sequential pathway, although unproductive off-pathway processes are observed. Application of relaxation dispersion methods yields unprecedented insights into the complex protein folding landscape of apomyoglobin.

ML-03 Order-disorder transitions in IscU, the scaffold protein for iron-sulfur cluster assembly and delivery

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In the ISU system, iron-sulfur clusters assemble on a highly conserved scaffold protein (IscU). We have found that IscU (both *Escherichia coli* and human) exists in solution in dynamic equilibrium between a structured state (S) and a disordered state (D). Exchange is slow on the NMR time scale, and the lifetimes of the states are on the order of 1 s. The equilibrium is temperature and pH dependent with maximal S state at about 25 °C at pH 8. We have identified single site amino acid substitutions in *E. coli* IscU that shift the equilibrium either toward the S or D state. Iron-sulfur cluster assembly takes place with IscU bound to a cysteine desulfurase (IscS), which generates sulfur by converting cysteine to alanine. Our NMR studies show that IscS acts as an IscU unfoldase. At sub-stoichiometric concentrations, IscS increases the hydrogen exchange rates of protected backbone amides of IscU. In addition, the rate of the S $\rightarrow$ D transition of apo-IscU is increased from 0.77 s^{-1} to 1.1 s^{-1} by the addition of 0.1 equivalent of IscS. In the complex between IscU and IscS, most of the residues of IscU are dynamically disordered. We examined the functional significance of this order-to-disorder transition by following the iron-sulfur cluster assembly reaction *in vitro* with wild-type IscU and with IscU variants with single amino acid substitutions at conserved residues that favor either the S or D state. Wild-type IscU assembled clusters most efficiently, followed next by the more disordered variants, although their clusters were less stable. By contrast, variants that favor the ordered state, such as (N90A), assembled clusters by a biphasic reaction with an initial slow step that we attribute to the order-to-disorder transition required to initiate sulfur transfer. After iron-sulfur clusters are assembled on IscU to form holo-IscU, they are transferred to acceptor proteins, such as apo-ferredoxin. This process is catalyzed by HscA, a specialized Hsp70-type chaperone, in an ATP-dependent reaction. NMR analysis of HscA revealed that it exists in equilibrium between two conformational substates. Upon binding IscU, HscA adopts a new single conformational state, but IscU becomes nearly fully disordered. By contrast, the highly stable variant of IscU (N90A) resists unfolding upon interaction with HscA. When HscA rebinds ATP, IscU is released from the complex and refolds. We conclude that IscU has evolved to undergo an order-to-disorder transition upon binding IscS that enables sulfur transfer during cluster assembly but also to retain a measure of structural stability to protect the cluster once it is formed. IscU undergoes a second order-to-disorder transition triggered by protein binding as a mechanism for iron-sulfur cluster release and transfer.

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Protein-protein interactions, Hsp 70-type chaperone, Unfoldase

ML-04 Applications of Stable Isotopes for Protein NMR

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The isotopic labeling of proteins is the fundamental basis of current NMR methodology, and almost all NMR studies are performed with isotope-labeled samples. The stereo-array isotope labeling (SAIL) method has been developed over the years by our group to overcome many of the existing difficulties in analyzing NMR spectra of proteins, by establishing a complete stereospecific and regiospecific pattern of stable isotopes that is optimal for acquiring the necessary information for structural determinations.[1)] The SAIL method has been successfully applied for structure determinations of relatively large proteins, which are difficult to assess by conventional NMR methods.[2,3)] For these applications, we have to prepare protein samples exclusively composed of SAIL amino acids, primarily by cell-free protein expression systems. This might impose a further barrier for promoting the SAIL method in the NMR community. However, for the various other applications of SAIL amino acids to address the local conformations and dynamics of selected amino acid residues, in many cases the usual cellular protein expression systems can be employed to prepare the NMR samples. We have been investigating various possibilities for using selective SAIL proteins to study the structures and dynamics of proteins and protein complexes.[4,5)]

In this presentation, I will describe some of our recent results on the application of selective SAIL proteins for studying aromatic ring flipping, proton exchange rates of hydroxyl and sulfhydryl groups, disulfide bond isomerization, etc. We are also trying to apply the selective SAIL method to very large proteins. A perspective on the SAIL-related methods along this direction will be given.

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Isotope Labeling, SAIL, Protein NMR

ML-05 “What’s next in fMRI/MRI?”

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Human size super high field MRI, especially at 7T, has been producing high resolution brain images at many of major MRI centers. Although such field strength is rather low to those in this NMR community, non invasive human brain imaging shows unprecedented beautiful anatomical structures. Further higher field instruments are about to become operational. Functional MRI at these high fields certainly has advantage because of their sensitivity and of the higher spatial resolution which gives better contrast to noise ratio presumably by better filling factor or less partial volume effect in small voxels. In the future, it will be possible to map neuronal assemblies of a few hundred micron size, each of which has its very specific functional character.

Functional MRI in these days is not only for studying evoked brain activities throughout the brain to understand the functional network, but also used to examine the phenomena at resting states of the brain. To study so called Resting State Network is getting very popular because fMRI signals in the brain show correlation at various sites to form functional networks which are very similar to those seen in evoked activities. Furthermore it has been shown that such RSN does vary with CNS disorders. These findings have led this RSN study to become the most popular major approach to learn the state of the brain.

Another popular fMRI approach is MVPA, multi-voxel pattern analysis, which claims to be information-based fMRI, not the one based on mere activation mapping. FMRI, usually BOLD fMRI, is based on the phenomena coupled to synaptic activity of neuronal assembly, and therefore it can pinpoint the event of the neuronal assembly but carries no information on the content of the neuronal processing activity. In MVPA, the patterns of voxels with fMRI signals can be assigned for the information of the task given to the brain by training with many trials. Then a new task can be tested and the pattern can be assigned to one with the information predetermined by the training. Each voxel is a feature point the task has and a pattern made out of these voxels can represent the content of input information. Because of the statistical power, single subject response becomes usable. That may open a way to deal with single individual’s measurements, which will be very important for clinical purpose or any topics which the uniqueness of individual response rather than a population average is important.

Up to now, fMRI experiments are essentially all based on BOLD (blood oxygenation level dependent) signal. It has many features which include non-invasiveness, tight coupling to neural events and has reasonably good sensitivity and high spatial resolution. However, it is a secondary phenomenon induced by vascular changes. The main short coming is the very slow response time of seconds instead of sub-second neuron-system does proceed.

Then, is there any non-BOLD fMRI which can replace BOLD based method? So far a few new methods have been presented. They are diffusion-gradient sensitized fMRI, white matter activation, near solid state water fMRI and e-current induced fMRI. There are methods which bring to us completely new aspects of functional responses in the brain. I like to mention these approaches here because they may draw quite interests from this community of NMR.

ML-06 The Importance of the Fourier Transformation in Spectroscopy. From Monsieur Fourier's Calculus to Medical Imaging

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The lecture exemplifies the relevance of the Fourier transformation in science. Its importance is fundamental to any experimental exploration where input-output relations are being exploited, spectroscopy being the foremost example. Experimental results, obtained in time-domain experiments, need to be transformed into the frequency domain for comprehension, and data from momentum space investigations require a transformation into the geometric space for visualizing images.

Fruitful applications are plentiful. The first practical usage of the Fourier transformation in spectroscopy took place in optical interferometry, starting with the seminal investigations by A.A.Michelson. Later, magnetic resonance, especially NMR, profited enormously from applications of the Fourier transformation. Molecular and medical imaging experiments, using x-rays and magnetic resonance are today among the most prominent applications of the Fourier transformation. Particularly promising is functional magnetic resonance (fMRI) for the better understanding of brain functions. Without the advent of the Fourier transformation, many applications of spectroscopy would not have become feasible. Of major importance is optimization of experimental performance, especially the dramatic increase in sensitivity that became possible. A survey on the exciting applications of the Fourier transformation in spectroscopy is presented.

Keywords: Analytical Spectroscopy, Fourier Transformation, Medical MRI

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Journey of in vivo NMR to MRI – special focus on T_2 and iron in human brain

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I started my NMR career in 1975 in Professor Fujiwara's laboratory, where Yoji Arata introduced me to the field. In the 1970s in vivo NMR emerged as a new field of NMR to investigate living processes in cells, organs, and individual humans noninvasively. I jumped into this field in 1978 when I was appointed to be a researcher at the National Institute for Environmental Studies. Throughout my career I have been attracted to in vivo NMR and the related emerging field of MRI. The meeting of International Council on Magnetic Resonance in Biological Systems held in Nara in 1978 was very stimulating to me as well as its successive meetings held bi-annually. I started with erythrocyte metabolism [1], and went on to photophosphorylation process in chlorella cells [2]. In 1980 Sir Rex Richards visited our institute and graciously spent almost all his time in discussion with me. This led me to visiting Biochemistry laboratory in Oxford from 1984 to 1985. There I was involved in studies of ATP production process in E. coli [3] and ischemic rat brain [4]. After coming back to Japan I gradually moved on to studies of skeletal muscle, liver, and brain in experimental animals related to metal toxicity and other environmental effects [5-7]. I obtained a 4.7T wholebody MRI in 2000 to work with structure and function of human brain.

In the following six years I focused my work on T_2 relaxation and iron concentrations in human brain. As is well known, T_2 is one of the most valuable contrast sources for MRI. It is routinely used for diagnosis for various diseases and for detecting in vivo processes like brain activation [8]. Iron on the other hand is an essential metal for life but an excess amount causes harmful effects through the generation of reactive oxygen species [9]. By collaborating with Mike Garwood in Minnesota I designed the MASE (Multiecho Adiabatic Spin Echo) sequence which allows precise measurements of T_2 in the brain [10]. Because of the long RF pulses during the echo spacing (~50%) the obtained T_2 is a mixture of T_2 during free precession and $T_{2\rho}$ during adiabatic refocusing pulses. Thus, we call the obtained T_2 an "apparent" transverse relaxation time ($T_2^\dagger$). I found that the $R_2^\dagger$ (= 1/ $T_2^\dagger$) in six brain regions can be described as a linear combination of the regional non-hemin iron concentration ([Fe]) and the macromolecular mass fraction (f_M) defined as 1 – water fraction [11]. Accordingly, $R_2^\dagger = \alpha[Fe] + \beta f_M + \gamma$, where coefficients α, β, and γ were experimentally determined using least square fitting by multiple regression analysis. We conducted this analysis for the first time with data obtained from 54 healthy subjects at 4.7T. We extended the work to other magnetic fields (B_0) to confirm the validity of the equation in general. For the lower field of 1.5 and 1.9T we made measurements with the same system by lowering the field strength of our magnet, and for 3 and 7T we did the measurements using Siemens Magnetom systems in Minnesota University. Then, we found the same equation was applicable to $R_2^\dagger$ with varying coefficients. Furthermore, it was found that α, β, and γ uniquely dependent on B_0. α is linearly dependent, while β is qaudratically dependent on B_0. The linear dependence of α is exactly the same as that observed for the water protons in a solution of ferritin [12]. The quadratic dependence of β is accounted for by a proton exchange model between bulk water and tightly bound water to macromolecules as well as exchangeable protons of macromolecules [13]. These findings gave us

In vivo NMR, MRI, Relaxation mechanism

an important clue to understand how transverse relaxation is determined in the living human brain. Finally, I developed a method for mapping the regional distribution of nonhemin iron in the brain. The obtained map exhibited dramatic differences in brain iron distribution for a patient with aceruloplasminemia [14].

On this occasion, I would like to thank all the people who taught me, encouraged me, collaborated and travelled with me in conducting these works throughout my science journey.

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IL-01 NMR of Natural Products at the Nanomole-Scale

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Structure elucidation of natural products (NPs) is highly dependent upon integrated spectroscopic techniques, particularly NMR and MS. Advances in NMR technology, particularly capillary probes and microcryoprobes[1,2,3] have expanded the mass sensitivity for investigations of NPs down to only a few μmole. Commercial NMR microcryoprobes offer the convenience of tube sample handling and exquisite sensitivity of inverse-detected $\{^{1}H\}^{13}C,^{15}N$-2D NMR experiments.[3] Such increased limits of detection now allow exploration of compounds from rare organisms, including uncultureable microbes and single specimens of invertebrates.[2] In this talk, several case studies from our laboratories within the past 3 years will illustrate the power of microcryoprobe NMR and the transformative dimensions revealed by the ability to fully characterize complex molecules when the 'world's supply' is only 1-8 nanomole.

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Natural product, Heterocycle, Microcryoprobe

IL-02 Structural insights into the binding of AIP4 WW2 domain and LMP2A PY motifs

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EBV is present in all population, infecting more than 95% of human beings, and EBV infection persists asymptomatically during the host's life. Although the precise role of EBV in cancer is not clear, it can cause several human tumors including Burkitt lymphoma, Hodgkin's diseases, nasopharyngeal carcinoma (NPC). EBV infection is latent in most cells, and EBV latency is maintained by the latent membrane protein (LMP) 2A, which mimics the B-cell receptor (BCR) and perturbs BCR signaling .The interactions of PY motifs of LMP2A with WW domains of Nedd4 family ubiquitin-protein ligases result in the ubiquitination of LMP2A-associagted proteins, and the subsequent down-regulation of B-cell signal transduction. Here we have solved the solution structure of WW2 domain of hAIP4, and investigated the binding mode of the LMP2A NTD and the WW2 domain. The WW2 domain shows typical WW structure with a three-stranded anti-parallel β-sheet, and binds to two PY motifs with different binding manner. Our NMR titration and ITC data demonstrate that the PY motifs of LMP2A can recognize and interact with the XP grove of WW2 domain (residues located around the third β-strand) weakly, and then the residues between two PY motifs optimize the binding by interacting with other region of WW2 domain widely. These weak but wide interactions can stabilize the complex form of PY motifs and WW domains. In addition, the hairpin loop region between β1 and β2 and especially the residue Val_{15} would play a critical role to distinguish the N-terminal and C-terminal PY motifs with different affinity.

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IL-03 Characterizing Backbone and Side-Chain Mobility by Solid-State NMR

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Accurate measurements of one-bond dipolar couplings in solid-state NMR are of great interest in the context of characterizing backbone and side-chain dynamics in biological molecules [1]. The partial averaging of dipolar couplings gives information about the amplitude of the motional processes from the fastest time scales up to the time scale corresponding to the inverse of the coupling strength, i.e., typically up to tens of microseconds. In the case of isotropic motions, the amplitude is often expressed in terms of an order parameter that characterizes the scaling of the dipolar coupling. However, no information about the actual time scales can be obtained from such measurements. Information about the time scales is available from relaxation data that can be used to supplement the information obtained from dipolar-coupling measurements. In principle, relaxation data measured at different B_0-field strengths allow a separation of time scales and motional amplitudes but due to the weak dependence of the relaxation-rate constants on the magnetic field strength such a determination is often not very precise for experimental data sets. Dipolar couplings are, thus, a very useful complement to relaxation data, and are generally required if motional amplitudes and time scales are to be analyzed quantitatively.

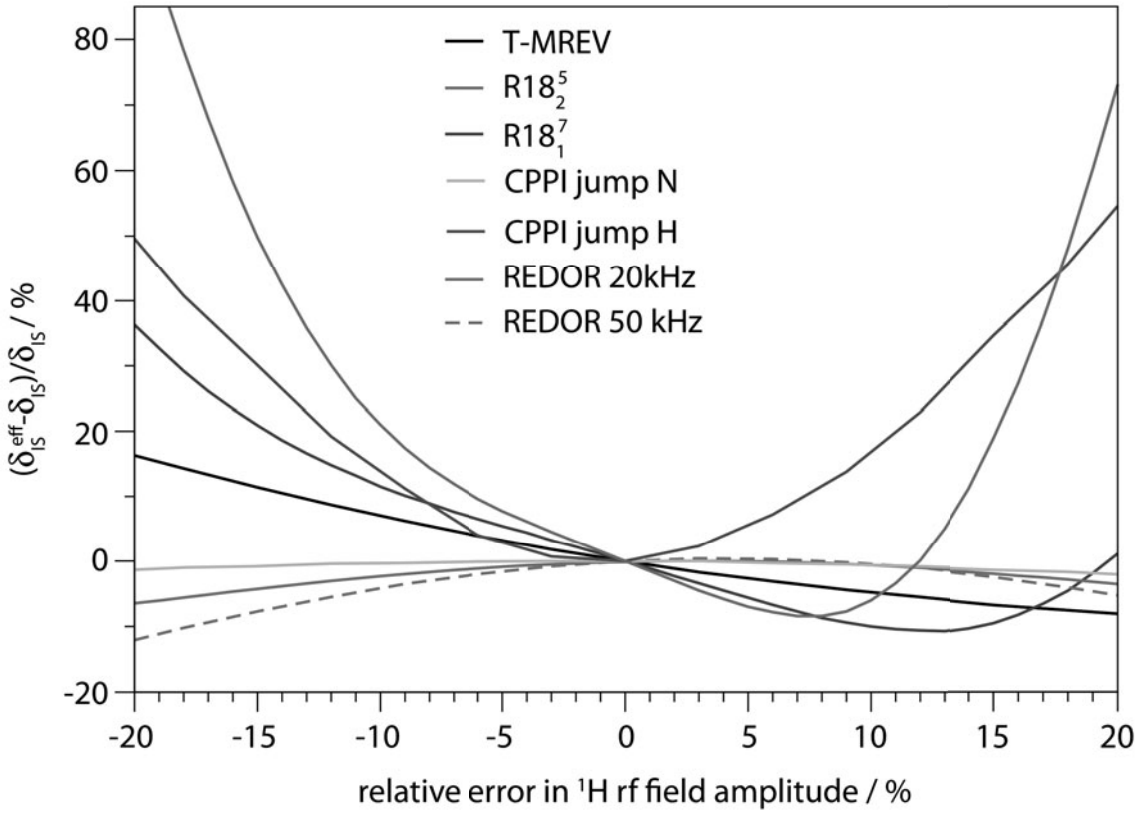

Figure 1: Dependence of the measured dipolar-coupling constant on the missetting of the ^{1}H radio-frequency field for various experimental schemes.

The accuracy of the experimental measurement of dipolar coupling measurement is crucial, especially if motional amplitudes in different proteins are to be compared, or if dipolar couplings are used in combination with relaxation data in order to describe time scales and amplitudes quantitatively. Therefore, we assess here the accuracy of different recoupling experiments by determining the magnitude of systematic errors that arise from miscalibrations of the radio-frequency amplitude (see Fig. 1), the influence of homonuclear dipolar couplings, chemical-shift offsets and CSA parameters, and the combined effect of miscalibrations of rf fields and homonuclear dipolar couplings.

We decided to investigate five different pulse schemes, namely the CPPI scheme, the $R18_1^7$ and $R18_2^5$ pulse sequences, the T-MREV sequence and the REDOR scheme using analytical calculation of the effective Hamiltonians and numerical simulations. From the numerical simulations we show that the REDOR scheme which can be used for a wide range of spinning frequencies gives the lowest systematic errors for samples with low proton density (deuterated samples). For samples with dense proton coupling networks (e.g., non-deuterated protein samples), the T-MREV sequence can be used at slow to intermediate MAS frequencies.

Motionally averaged dipolar couplings are often analyzed in terms of order parameters. However, only isotropic motions lead to a scaling of the dipolar coupling that can be decribed by a single parameter. Anisotropic motions will lead, in general, to asymmetric effective dipolar couplings that have to be characterized by the anisotropy and the asymmetry parameters. We show that such asymmetric dipolar couplings can be measured when using the REDOR pulse scheme. Asymmetric dipolar couplings are especially important for the description of side-chain motions where not all rotameric conformations are populated with the same probability. We show examples for the measurements of backbone and side-chain mobility using the REDOR scheme.

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solid-state NMR, dipolar-coupling measurement, dynamics, side-chain rotamers

IL-04 New NMR Experiments for Challenging Proteins

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While NMR assignment and structure determination of small proteins has become near routine it has still remained a time consuming procedure for larger proteins. Thus, we have made much effort to render this process more efficient and fill holes that could not be covered with traditional approaches. This includes double TROSY hNcaNH experiments that use the TROSY effect both at the preparation and detection ends of pulse sequences [1]. More recently, we discovered that a slight modification of the experiment yields patterns that make assignment extremely efficient [2], and we could assign a 38 kDa protein in three days.

On the other hand, we have made efforts to explore ^{13}C and ^{15}N direct detection for assigning difficult proteins, such as regulatory protein regions containing many prolines and phosphorylation sites. Due to the slow transverse relaxation of carbon and nitrogen signals, pulse sequences become very efficient and compensate for the low inherent sensitivity [3-5].

Resolution and sensitivity of these experiments can be enhanced dramatically with non-uniform sampling and suitable processing methods. This allows using the resolution power of modern high-field instruments in multidimensional NMR experiments. This is in contrast to uniform sampling where only a small part of the indirect dimensions can be covered, which largely underutilizes the power of state-of-the-art instruments. Processing methods for non-uniformly sampled data have advanced dramatically so that high-resolution sparsely sampled spectra up to three indirect dimensions can be processed within a few hours. Application to large protein systems, such as non-ribosomal peptide synthetases and complexes of translation initiation factors will be shown.

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YIL-01 Side-chain dynamics of hydrogen bonds and ion pairs

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One of our long-term goals is to understand how the kinetic aspects of protein functions are linked to side-chain dynamics involving formation and breakage of hydrogen bonds and ion pairs. As the first step to achieve this long-term goal, our group has started developing methods for characterizing side-chain hydrogen bonds and ion pairs.[1-5] We chose lysine side-chain NH_3^+ groups as a subject of our methodological development because lysine side chains play an important role in protein-DNA interactions, which our group is interested in. To study lysine side chains, we have successfully developed NMR techniques for minimizing adverse effects of scalar relaxation arising from rapid hydrogen exchange,[1] for facilitating resonance assignment,[1,2] for characterizing protonation and deprotonation,[2,3] and for characterizing the side-chain dynamics.[4,5]

To investigate the dynamics of lysine NH_3^+ groups, we developed two different kinds of methods: one based on ^{15}N relaxation,[4] the other on long-range ^{15}N-^{13}C scalar coupling.[5] With implementation of the HISQC principle to minimize effects of scalar relaxation due to hydrogen exchange,[1] our ^{15}N relaxation methods permit measurements of ^{15}N relaxation parameters for lysine NH_3^+ groups. To analyze ^{15}N relaxation data for $^{15}NH_3^+$ groups, we modified the theoretical framework developed previously for $^{13}CH_3$ groups. This permits determination of order parameters, correlation times for bond rotation and reorientation of NH_3^+ groups occurring on a ps–ns timescale (Fig. 1). We also developed the NH_3^+-selective ^{15}N-relaxation-dispersion experiment that permits investigations of slower dynamics on a μs–ms timescale.[4] In another work, we developed NH_3^+-selective $^1H/^{13}C/^{15}N$ heteronuclear correlation experiments to measure two different types of long-range ^{15}N-^{13}C *J*-coupling constants: one between intra-residue $^{15}N\zeta$ and $^{13}C\gamma$ nuclei ($^3J_{N\zeta C\gamma}$), and the other between $^{15}N\zeta$ and carbonyl $^{13}C'$ nuclei across a hydrogen bond ($^{h3}J_{N\zeta C'}$).[5]

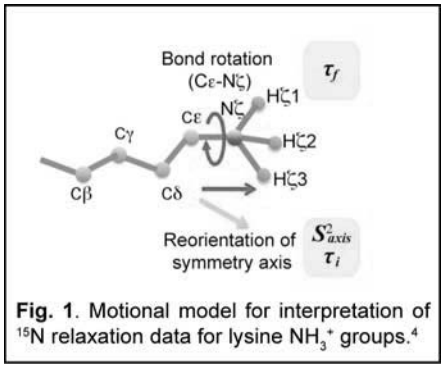


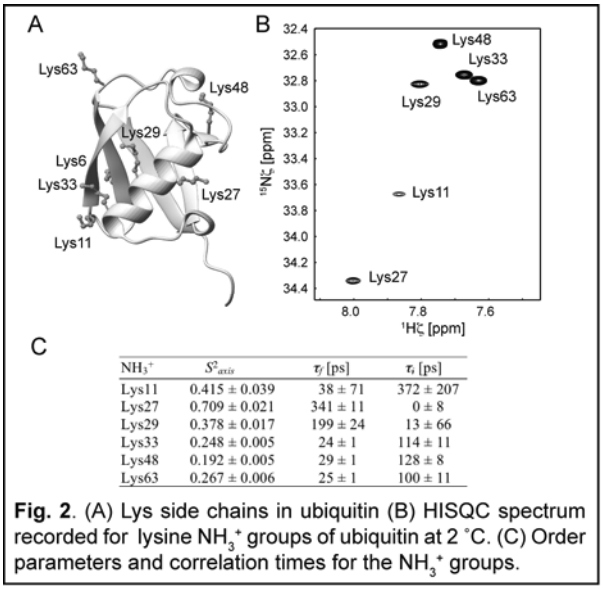
Fig. 1. Motional model for interpretation of ^{15}N relaxation data for lysine NH_3^+ groups.[4]

By applying these methods, we studied the dynamics of lysine NH_3^+ groups of ubiquitin (Fig. 2). In collaboration with Prof. Rafael Brüschweiler's group (Florida State University), we compared our experimental order parameters for the lysine NH_3^+ groups with those from a 1-μs MD simulation, and found good agreement between the two. From the ^{15}N-relaxation-dispersion experiment for lysine NH_3^+ groups, slower dynamics occurring on a ms timescale have also been detected for Lys27. Furthermore, both the MD simulation and the experimental correlation times for the bond rotations of NH_3^+ groups suggest that their hydrogen bonding is highly dynamic with a sub-nanosecond lifetime. The NH_3^+ groups of Lys29 and Lys33 exhibit measurable $^{h3}J_{N\zeta C'}$ couplings arising from hydrogen bonds with backbone carbonyl groups of Glu16 and Thr14, respectively, although their order parameters indicate high degree of internal motions (Fig. 3). When interpreted

Keywords: Side-chain dynamics, hydrogen bond, ion pair

together with the $^{3}J_{N\zeta C\gamma}$ coupling constants and NMR relaxation derived S^2 order parameters of the NH_3^+ groups, the data strongly suggest that hydrogen bonds involving NH_3^+ groups are highly dynamic and undergo transitions between locally associated and dissociated states on a sub-nanosecond timescale.[5]

Our recent data on the dynamics of intermolecular ion pairs between protein and DNA will also be demonstrated at the symposium.



NH_3^+	S^2_{axis}	τ_f [ps]	τ_s [ps]
Lys11	0.415 ± 0.039	38 ± 71	372 ± 207
Lys27	0.709 ± 0.021	341 ± 11	0 ± 8
Lys29	0.378 ± 0.017	199 ± 24	13 ± 66
Lys33	0.248 ± 0.005	24 ± 1	114 ± 11
Lys48	0.192 ± 0.005	29 ± 1	128 ± 8
Lys63	0.267 ± 0.006	25 ± 1	100 ± 11

Fig. 2. (A) Lys side chains in ubiquitin (B) HISQC spectrum recorded for lysine NH_3^+ groups of ubiquitin at 2 ˚C. (C) Order parameters and correlation times for the NH_3^+ groups.

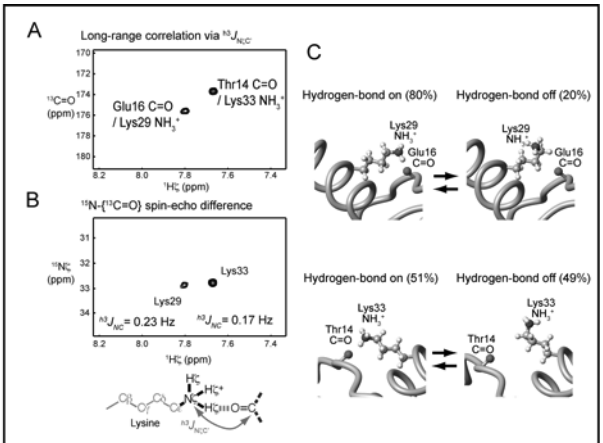


Fig. 3. (A, B) Spectra recorded for measuring $^{h3}J_{NC'}$ constants for lysine NH_3^+ groups of Lys29 and Lys33. (C) Formation and breakage of hydrogen bonds seen in a 1-μs MD trajectory. The average lifetimes of the hydrogen bonds in the MD simulation were 45 ps for Lys29 and 30 ps for Lys33.

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YIL-02 Structure of the CCA-bulge region of prohead RNA of bacteriophage phi29

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DNA packaging motors of bacteriophages are among the most powerful biological motors described, generating forces of 50-60 pico newtons. Prohead RNA (pRNA) is an essential structural component of the DNA packaging motor of bacteriophage phi29. pRNA forms a pentameric ring structure bound at the packaging vertex of the prohead capsid, where it interacts with the five-fold head shell (gp8 protein), the dodecameric connector (gp10 protein), and the pentameric packaging ATPase (gp16 protein). Deletion of nucleotides from the CCA bulge region of pRNA modifies the DNA-packaging activity of the motor as deletion of the bulge resulted in no DNA packaging in a defined in vitro packaging system. We solved the solution structure of a 27mer RNA fragment containing the CCA bulge (b27). We found that the bulge spans four nucleotides (17-UCCA-20) instead of the predicted three nucleotides (18-CCA-20). Mutational analysis confirmed that U17 was important for DNA packaging activity as well as the CCA nucleotides. The bulge introduces bending of the helical axis by 33 degrees. RDC data analysis indicated that the inter-helical motion around this bend was smaller than other known bulge containing RNA structures, suggesting this bend may be important for pRNA functions. Cryo-EM 3D reconstructions of proheads containing pRNA with or without the CCA bulge confirmed that the bulge-induced bend was preserved in the prohead-bound RNA. We generated a structural model of the 120-nucleotide pRNA by connecting the b27 NMR structure with a recently published crystal structure of the pRNA prohead binding domain that lacks the A-stem containing the bulge. This 120b pRNA model structure was fitted into a cryo-EM map of proheads to generate a pentameric pRNA structural model. The gp16 binding region of pRNA has been identified, and it included 17-UCC-19. The pentameric pRNA structural model showed that 17-UCC-19 were facing inside and ready to interact with gp16. ATP consumption and DNA packaging efficiency were tested with variants of pRNA containing mutations in the bulge region. Although the prohead containing these mutant pRNAs could hydrolyze ATP, some mutants such as deletion of CCA did not package any DNA. Interestingly, there was no DNA packaging intermediates found using proheads containing the bulge-mutant pRNA. In other words, once DNA packaging started, it completed packaging all the DNA. Therefore, we speculate that the bulge-induced angle is important for pRNA to align the gp16 pentamer in a proper position to initiate DNA packaging.

Prohead RNA, DNA packaging motor, pentamer model

YIL-03 Stable Isotopic Labeling of Proteins for Biomolecular NMR in Post-structural Genomics Era

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While structure determination of isolated domains has been tremendously advanced, understanding of domain-domain interactions in a multi-domain protein is lacking behind. NMR is an ideal tool for characterizing transient interactions between domains. However, NMR analysis of multi-domain proteins suffers from signals overlap as well as short T_2 relaxation times because proteins often contain a large number of atoms, multiple domains, and disordered regions. We have advanced segmental isotopic labeling protocols and used biosynthetic approaches for accelerating NMR analysis of intact proteins.

Stable isotopic labeling is almost prerequisite for any NMR analysis of proteins. Uniformly labeled proteins have been routinely prepared from bacterial expression systems, thereby significantly advancing NMR analysis of biomolecules by expanding dimensions of NMR spectra with triple-resonance experiments. NMR analysis of larger proteins (>30kDa) still suffers from signal overlaps even with multi-dimensional NMR spectroscopy. This overlap problem is an inherent problem associated with larger molecules, which is still difficult to be resolved even at the highest magnetic field currently available. There has been significant effort towards reducing signal overlaps since the beginning of biomolecular NMR. Nowadays, it has become feasible to introduce very sophisticated labeling strategies such as stereo-array labeling and site-specific labeling although these techniques are not readily accessible to non-experts. We are interested in simple and inexpensive labeling techniques that can be achieved without special requirements such as cell-free protein synthesis or special chemicals. In addition to uniform isotopic labeling, selective amino-acid labeling has been often used to reduce the number of NMR signals in proteins. Selective amino acid labeling can be easily achieved by feeding labeled amino acids into growth media. However, selective amino-acid labeling can solve the signal overlaps but leaves the problem of sequence specific resonance assignments. Moreover, structural genomics projects around the world has speeded up structure determination and significantly contributed the number of determined structures in the database. *de novo* NMR structure determination is thus increasingly become time-consuming and expensive when structures of homologues determined by X-ray and NMR are available. The number of determined structures will continue increasing, making it more likely to find a structure in the database in the future. High-throughput approach in the structural genomics projects has also been focusing on self-contained domain that are excised from the full-length proteins to reduce their molecular weights to a manageable size and that provide high quality NMR spectra. However, many proteins, including cellular signaling proteins, cell-surface receptors, and large enzymes, are often constructed from a few modular domains, which are structural and functional units, and connecting linkers. Minimization approaches for structural analysis might neglect functional aspects of a domain in an intact protein and may not represent all aspects of the structure-function relationship in the full-length context. While the structure determination of individual, isolated domains has been tremendously accelerated, understanding of domain-domain interactions within a multi-domain protein is lacking behind. Structural analysis of intact proteins is still challenging. To advance structural analysis of proteins in intact contexts, we have been developing novel labeling approaches including segmental isotopic labeling. Segmental isotopic labeling is one of the promising approaches for larger

proteins originally developed by Yamazaki et al., with which only a segment of a protein is isotopically labeled [1]. Segmental isotopic labeling does not only alleviate the overlap problem but also retain the possibility to perform sequence specific resonance assignments by conventional triple-resonance experiments. Despite high potential of segmental isotopic labeling, it has not been widely used presumably because it is a labor-intensive procedure. In the past years, we have improved segmental isotopic labeling protocols by introducing a time-delayed dual expression system in which none of precursor proteins needs to be purified, thereby simplifying the procedure tremendously [2]. We also extended this approach to a central fragment labeling by three-fragment ligation using an engineered highly efficient protein splicing domain [3]. Although there are still some limitations, segmentally isotope-labeled sample can be routinely produced in less than a few days [4].

Another severe problem associated with larger proteins is short transverse relaxation times, deteriorating the quality of NMR spectra. Many sophisticated triple resonance experiments still suffer from short transverse relaxation times even with TROSY approaches. The bottleneck of sequence specific assignments often originates from ambiguities in identification of amino acid-types of spin systems, which currently relies on spin topology and chemical shifts. We introduced a simple approach to use biosynthetic pathways to classify residue-types, complementing the existing approaches. Since the information of amino acid types can be extracted from simple and sensitive experiments such as HNCA, HNCO, the use of biosynthetic pathways for isotopic labeling is less prone to the molecular sizes. The combination of segmental isotopic labeling and biosynthetic labeling could accelerate NMR analysis of large intact proteins, which is currently challenging for NMR spectroscopy as well as X-ray crystallography.

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Segmental isotopic labeling, intein, metabolic labeling

YIL-04 Misfolding Reactions and structures in self-assembled Alzheimer's β-amyloid and graphene-related carbon nano-materials

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Two separate topics using solid-state NMR (SSNMR) will be discussed. In the first topic, we discuss structures and functions of nano-structured Alzheimer's β-amyloid (Aβ) peptide in amyloid fibrils and other aggregates, major suspects in Alzheimer's disease. By ^{13}C and ^{15}N SSNMR, we examine detailed metal coordination structure of Cu(II)-bound 40-residue Aβ in fibrils, which has been suspected to produce reactive oxygen species such as H_2O_2, for the first time.[1] Furthermore, it is shown that SSNMR is a sensitive probe of redox-reactions associated with Cu(II)/Cu(I)-Aβ complexes, which produces H_2O_2 at an elevated level. Other subjects such as structure of diffusible amylod aggregates and protein structure during solid-phase synthesis will be discussed. In the second topic, we discuss novel SSNMR approaches for characterizing structures of graphene/graphite-based nano-materials, which include chemically modified graphene. It is shown that multi-dimensional SSNMR on ^{13}C-labeled graphite oxide (GO), a notable precursor for mass production of a single layer graphene, provides detailed information on its complex chemical connectivity.[2,3] We also examine structural changes after chemical reduction of GO with ^{15}N-labeled hydrazine. Based on the triple-resonance SSNMR analysis, we propose possible new graphene-edge structure in GO and chemically reduced GO. Such chemical modifications at the edge of graphene sheets potentially allow us to alter the functionalities of graphene based systems. The motivations and advantage of SSNMR-based studies for graphene based system will be discussed.

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Keywords: Alzheimer's beta, Solid-state NMR, Copper-binding