NMR studies of folded and unfolded proteins
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Summary and Future Perspectives

Solution state NMR spectroscopy is the most powerful technique to study folded and unfolded proteins in atomic details. Through the utilization of this technique, we can obtain comprehensive information involving structures, dynamics, electrostatic interactions, and protein-protein interactions, which are very essential for comprehensive understanding of many biological mechanisms at atomic details. Tremendous and rapid developments in NMR methodologies push the size limit to study folded proteins, accelerate the protein NMR measurement, and overcome the challenging problem to assign the highly overlapping signals of the intrinsically disordered proteins (IDPs).

In this dissertation, we studied both the electrostatic interactions of folded protein, photoactive yellow protein (PYP), and conformation detail of soluble unfolded amyloid-β peptides. We also developed the NMR methodologies: (i) a new approach to determine Tyr sidechain pKa value in folded protein (ii) a simple and tunable way to speed up NMR measurement of intrinsically disordered proteins using solvent paramagnetic relaxation enhancement (PRE).

Comprehensive determination of side chain pKa values of PYP: method development and explanation of strong pH dependence of ground state recovery PYP

We presented an NMR approach based on 2D Cγ-Hβ correlation spectroscopy to determine the pKa values of individual Tyr side chains in native PYP proteins with high sensitivity and resolution. This method provides a number of benefits over existing practices. First, it does not require mutagenesis to assign the NMR resonances; and secondly, it facilitates the complete and comprehensive analysis of electrostatic interactions of tyrosine side chain in proteins.

For PYP from *H. halophila*, we successfully determined the pH-dependence of the protonation states of all individual Tyr side chains using 2D CG(CB)HB experiment. In PYP, three classes of tyrosines were observed based on their titration behavior: solvent exposed Tyr-76 and Tyr-98); buried in the hydrophobic environment (Tyr-118); and, hydrogen bonded (Tyr-42 and Tyr-94), with pKa values of ~10, 12 and above 13, respectively. This study also demonstrated that the short hydrogen bonds between Tyr-42...
the $p$-coumaric acid ($p$CA) chromophore persists over the entire pH range where the protein is chemically and thermodynamically stable. Our results indicate that previous observation of pH-dependence changes in PYP photocycle kinetics cannot be caused by protonation state change in Tyr-42 in the ground state. This study implies that it is very unlikely that the $p$CA chromophore undergoes changes in its electrostatic interactions in the electronic ground state.

We found that out and back HB(CB)CG pulse sequence did not provide better sensitivity for recording $C\gamma$-$H\beta$ spectrum of fully protonated PYP due to long and lossy $^{13}$C magnetization transfer step. However, the sensitivity of recording NMR measurement of $C\gamma$-$H\beta$ spectrum can be improved by performing partial deuteration. This method might be useful for determining the side chain pKa of higher molecular weight protein. Moreover, to achieve better sensitivity of much higher molecular weight protein, 2D $^{13}$C detection experiment of fully deuterated of protein can be utilized. Indeed, using $^{13}$C detection experiment can consume longer experimental time since the sensitivity is proportional to $\gamma^{3/2}$ of detected nuclei. Fortunately, the tremendous advances in technology, which allow the use of cryogenic probe and much higher magnetic field, enable to improve the sensitivity of the NMR spectra.

We also performed comprehensive side chain pKa determination of PYP, particularly focus in the active site of PYP. This investigation aims at explaining strong pH dependence of ground state recovery PYP. We accomplished nearly complete assignments of backbone and side chain $^1$H, $^{13}$C and $^{15}$N resonance at pH 5.8 and 20 °C of PYP in its electronic ground (pG) state. Using this assigned chemical shifts, we monitored the pH-dependence of all titratable chemical shifts in PYP using 2D and 3D heteronuclear NMR experiments. NMR spectroscopy has been established as the most reliable method to determine the protonation state of individual titrable groups in proteins. Several 2D NMR experiments have been developed to determine the individual side chain pKa for titrable groups such as His, Asp, Glu, Lys, Arg and Tyr, which have been explained in chapter 2.

Our study demonstrated that none of the titratable group change their protonation state in the ground state. We showed that the side chain of Glu-46 and Tyr-42 form persistent
hydrogen bonds to the $pCA$ chromophore over the entire pH range. This finding implies that those bonds have persistent geometry in that pH range. Hence, the pH-dependence of PYP in the photocycle can only be explained by pH-dependence changes in the pB state.

Several studies suggest that the side chain of Arg-52 is protonated. However, neutron crystallography study shows that the side chain of Arg-52 is deprotonated. Our NMR data demonstrates that there is no change in the protonation state of Arg-52 in the pG state from pH 3.4 up to 11.2. We observe that the $^{15}$N chemical shift of Arg-52 guanidino group around 70 ppm, which would typically be interpreted that Arg-52 is protonated. However, previous study shows that the $^{15}$N chemical shifts of the terminal guanidine group depend strongly on the solvent chosen for measurements (Xiao and Braiman, 2005): a non polar environment leads to more shielding and hence a lower chemical shift. Hence, chemical shift data alone is not sufficient to enable for an unambiguous interpretation of the Arg-52 protonation state. Using another technique such as FTIR might provide more information which supports one of the study. Moreover, comprehensive determination of side chain pKa in the active site of PYP Arg52Ala might also explain why the bell-shaped profile of pH dependence of kinetic recovery of this mutant is similar with the wild-type but the rate of kinetic recovery is different. To confirm the presence of Low-Barrier-Hydrogen-Bond (LBHB) between chromophore $pCA$ and Glu-46 and Tyr-42, one can determine the pKa of chromophore $pCA$ unambiguously by incorporating labeled ($^{13}$C,$^1$H) chromophore $pCA$ into unlabeled PYP and monitor the chemical shift changes of carboxyl group as function of pH. Similar pKa between side chain of Glu-46 and chromophore $pCA$ can prove the existence of LBHB in PYP active site. Monitoring chemical shift in the pB state will be important to explain which residues involved in the pH-dependence of photocycle kinetic and what the corresponding pKa they have. To achieve this goal, one need to set up NMR experiments which facilitate blue light irradiation upon measurement. The results from this study also can be used to improve some parameters for theoretical calculation. One can use also the N-terminally truncated of PYP (Delta25-PYP) since it exhibits very similar photocycle as the corresponding wild-type PYP but the lifetime of pB state is much longer (Bernard et al., 2005)
Unstructured of soluble Amyloid β dimers leading to different aggregation properties

Non-fibrillar form of soluble amyloid-β (Aβ) is believed to play important role in Alzheimer’s disease (AD). The fact that soluble Aβ dimers are detected in the extract of Alzheimer’s human brains indicates that Aβ dimer may be the basic building block of AD associated with synaptotoxic species. Due to this reason, the information about the structural details and properties of Aβ dimers is very important to be explored in order to find clues to prevent, suppress, or even to cure Alzheimer’s disease.

In chapter 4, we investigated the aggregation properties and conformation details of Aβ monomer and two Aβ dimers: [Aβ (M1-40)S26C]<sub>2</sub>, which is Aβ (M1-40) containing Cys in place of Ser-26, providing disulfide cross-linked dimer; and [Aβ (M1-40)]<sub>tyr</sub>, which has a covalent dimerization at the side chain of Tyr-10. Our data showed that all three species of monomer and dimer Aβ (M1-40) aggregate at different rate and form different products. NMR results demonstrated that all three species of Aβ (M1-40) are fully unstructured and have little differences in structural propensities, which are correlated with their aggregation properties. Apparently, it may be sufficient to modulate structural propensities only slightly, thereby affect their aggregation properties and toxicity.

For further study, comparison of LTP measurement result for every species of Aβ monomer and dimer is very important as it provides insight into their toxicity. Producing several Aβ dimers which have disulfide covalent bond in different position (e.g disulfide covalent bond in first beta sheet region or in second beta sheet region) and investigating their aggregation properties, toxicities and conformation details might give valuable information about Aβ conformation related to their toxicities and aggregation properties.

Studying aggregation properties and conformation details of Aβ(M1–42) and the dimeric form of Aβ(M1–42) is also deep interests because the amount of Aβ42 is higher in the brain of AD patients. Since Aβ42 aggregates more rapidly than Aβ40, therefore the fast and sensitive NMR measurement for these peptides become necessary. Rapid NMR measurement of this fast aggregating peptide can be accommodated using neutral paramagnetic agent Fe(DO3A).
Metal ion, which enter the body via many sources, including drinking water, from foods such as shellfish, nuts, red meat and many fruits and vegetables, and also food supplement. Metal ions are found to promote (Bush et al., 1994; Mantyh et al., 1993) or hinder amyloidosis (Miura et al., 2000; Raman et al., 2005; Yoshiike et al., 2001). However, the interaction between soluble low n-oligomer (especially Aβ dimer) and metal ion and how these metal ion can promote or hinder amyloidosis are still poorly understood. Studying the interaction between Aβ dimer and metal ions might provide valuable information related to the mechanism of amyloidosis.

Several studies suggest that the presence of soluble low-n-oligomer Aβ species cause neuronal dysfunction and memory impairment in Alzheimer's disease rather than mature fibrils (Bucciantini et al., 2002; Walsh and Selkoe, 2007). Comprehensive information about aggregation processes in atomic details can be investigated by studying the aggregation and conformation details of more extended Aβ peptides (trimer, tetramer). The purpose of this study is to know what the most toxic of Aβ species are and their aggregation properties and conformation details.

Studying Aβ species as therapeutic target for Alzheimer's disease offers significant contribution for AD treatment. Previous study indicated that toxicity can be reduced by decreasing the amount of stable low-n-oligomer Aβ species. Therefore, such molecules which can accelerate the polymerization, might be important to reduce the neurotoxicity. A recent study demonstrated the acceleration of Aβ fibrillation through the action of orcein-related small molecule O4 (2,8-bis-(2,4-dihydroxy-phenyl)-7-hydroxy-phenoxazin-3-one) which binds to the hydrophobic residue of Aβ peptides and suppressed inhibition of LTP (Bieschke et al., 2012). However, it is still unclear whether this compound can promote the aggregation of Aβ peptides at nanomolar concentration in vivo. Similar case was shown by conserved protein called MOAG/SERF, which can speed up the aggregation kinetics of amyloid fibrilization (Falsone et al., 2012; van Ham et al., 2010). One important question is how this protein interact with Aβ peptides and promote the aggregation. Furthermore, it also needs to be investigated whether the presence of MOAG/SERF can suppress the inhibition of LTP in vivo.
Speed and sensitivity of NMR spectroscopy of intrinsically disordered proteins using solvent that paramagnetic relaxation enhancement

For more than a decade, intrinsically disordered proteins (IDPs) have attracted great interests due to their significant abundance in eukaryotic proteomes, with over 40% of human proteins containing long disordered regions, or being completely lack of structure under physiological conditions. IDPs are also known to play important roles in cellular processes such as molecular recognition, transcription, and replication. Moreover, they are also found to be vital in the development of numerous human pathologies, such as neurodegenerative diseases, cardiovascular diseases, and cancers.

NMR spectroscopy is the most suitable method to gain insight into the details of protein conformational disorder at atomic resolution. However, the NMR spectra of IDPs are highly crowded due to the inherently flexible nature of IDPs that composes of extensive conformational averaging, thus extremely reduces nuclear chemical shift dispersion. As consequences, spectral overlaps make the resonance assignment for these proteins become very challenging. To complete the unambiguous resonance assignment for unfolded proteins, high-dimensional spectra are needed, which require the sampling of the time evolution in every dimension. The acquisition of these spectra generally require very long recording time. It implies that excellent time-stability of the protein samples without aggregation should be achieved, which is a difficult requirement for IDPs. Therefore, it is of great interest to speed up the NMR recording time for IDPs.

In chapter 5, we demonstrated a fast and sensitive solution-state NMR data measurement for unfolded proteins using tunable extrinsic paramagnetic relaxation enhancement (PRE), Ni(DO2A). Due to the short electron-spin relaxation time of nickel (II), the neutral organometallic complex Ni(DO2A) was found to be particularly suitable for this goal. Addition of 30 mM of Ni(DO2A) caused the average proton relaxation rate for the intrinsically disordered protein α-synuclein to increase from 1.76 s⁻¹ to 5.46 s⁻¹ with negligible line broadenings or other changes to the NMR spectra.
By combining solvent PRE Ni(DO2A) and projection reconstruction techniques, we record a high-quality 3D HNCO spectra of \( \alpha \)-synuclein as little as 15 minutes. Our data demonstrated that the neutral paramagnetic agent Ni(DO2A) is accessible to the entire disordered polypeptide chain, which provides a significant advantage over its application to folded proteins.

In chapter 6, as an extension to our findings, we described an optimal paramagnetic relaxation agent for NMR spectroscopy of IDPs. We found the neutral high-spin iron chelate Fe(DO3A) can fulfill the criteria for biomolecular NMR applications involving intrinsically disordered proteins (IDPs). Rapid data acquisition is important for IDPs, as unfolded proteins are often prone to aggregation. We reported a five-fold acceleration for \(^1\)H detection NMR spectroscopy of intrinsically disordered human \( \alpha \)-synuclein, using only 4 mM Fe(DO3A). Furthermore, we obtained similar time savings for proton-less NMR spectroscopy of deuterated \( \alpha \)-synuclein, employing 20 mM Fe(DO3A). These results demonstrate the robustness of the usage of PRE to speed up the NMR measurement times.

Many IDPs, which involve in many important processes (e.g. neurodegenerative diseases), are not stable in the high concentration and they can aggregate easily. Application of Fe(DO3A) or Ni(DO2A) to NMR measurement of easily aggregating proteins or peptides will provide significant benefit on accelerating NMR recording time and increasing sensitivity enhancement. Co-solute PRE Fe(DO3A) or Ni(DO2A) offer simple and tunable way to improve the sensitivity and resolution of NMR spectroscopy. In addition, paramagnetic agent Fe(DO3A) and Ni(DO2A) are also can be applied to reduce the instrumental time of 5D NMR experiments of IDPs, which are useful to overcome the problem of assigning highly overlapping signals. As shown in chapter 6, one can combine solvent PRE with other methods (e.g. projection reconstruction) to reduce the NMR instrumental time.

Some biomolecules, such as unlabeled peptide, primary and secondary metabolite, and also natural products from plant or organisms, are extracted in very low yield. Depending on the size of the molecules, these paramagnetic agent offer an aid to improve the sensitivity and/or resolution of NMR measurement of those biomolecules by reducing T1 relaxation of the excited nuclei. To get benefit on reducing T1 relaxation with negligible

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line broadening, one should use paramagnetic agent which has electronic T1 relaxation shorter than rotational correlation time of the molecule. Moreover, in the presence of these paramagnetic agent one can also record 2D NMR experiment in shorter time to unambiguously assign unlabeled peptide.

NMR spectroscopy is also suitable technique to analyze metabolites quantitatively in biological samples. However, since T1 relaxation of every biomolecules varies a lot, therefore one need to apply long recycle delay between consecutive scans to make sure that all magnetizations of excited nuclei have returned back to equilibrium. These paramagnetic agents also can be applied for analyzing metabolites quantitatively using NMR spectroscopy. The choice of paramagnetic agent depend on the size of metabolites. For small molecules (few kDa), it is better to use Ni(DO2A) rather than Fe(DO3A) since the use of Fe(DO3A) only can create significant line broadening on the spectrum.

This dissertation provides not only insight into protein properties at atomic details, which are related to their biological system, but also knowledge about NMR method development, which can be useful to be applied for many biological systems.

Through the works done in this dissertation, we hope that this dissertation is not only beneficial for particular subjects but also can give valuable contribution for NMR community to develop NMR methodologies.

References


