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Advanced techniques of solution nuclear magnetic resonance spectroscopy for structural investigation of protein-protein interaction

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Abstract Investigation of the protein-protein interaction mode at atomic resolution is essential for understanding on the underlying functional mechanisms of proteins as well as for discovering druggable compounds blocking deleteriou interprotein interactions. Solution NMR spectroscopy provides accurate and precise information on intermolecular interactions even for weak and transient interactions, and it is also markedly useful for examining the change in the conformation and dynamics of target proteins upon binding events. In this mini-review, we comprehensively describe three unique and powerful methods of solution NMR spectroscopy, paramagnetic relaxation enhancement (PRE), pseudo-contact shift (PCS), and residual dipolar coupling (RDC), for the study on protein-protein interactions.

Keywords paramagnetic relaxation enhancement, pseudo-contact shift, residual dipolar coupling

Introduction

Great advantages of solution NMR methods come from the ability to determine complex structures and to reveal the intermolecular interaction site and mode between target molecules and their binding partners at the atomistic resolution in solution. Sepecifically, information obtained from NMR spectroscopy for weak interaction systems (~milli molar order of dissociation constant (K_d)) which are otherwise difficult to be detected have contribured to our deeper understanding of the mechanistic moldel of functions of biomolecuels including proteins and nucleic acids. The elucidation of structural properties of protein-protein interactions in solution have offered direct and fundamental insights into the mechanisms of numerous biological processes and the development of new drugs.

Broadening and overlapping of NMR signals of target molecules with high molecular weights often limited the in-depth study and application of solution NMR: however, recent advances in the NMR technique and sample preparation have casted light on overcomimg these difficulties and thus expanded application of solution NMR to the study on a variety of interprotein interactions. There are several convenient and popular NMR experiments as well as analyses for investigation of the protein-protein interaction, such as chemical shift perturbations, changes in the NMR peak intensity and NOE-based techniques (e.g. cross-saturation or isotope-filtered NOESY). In this review, we briefly introduce the key

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and unique recent methodologies of solution NMR spectroscopy for examinning protein-protein interactions by focusing on PRE, PCS and RDC. We address the basis of each method and provide practical tips for the experiment, sample preparation, and anlysis.

Paramagnetic effects, PRE and PCS

PRE and PCS - Dipolar-dipolar interactions between a center of paramagnetic origin and NMR-active nuclei (e.g. ^{1}H, ^{13}C and ^{15}N) enhance transverse relaxation of magnetizations , so-called paramagnetic relaxation enhancement (PRE): The PRE effect causes remarkable attenuation of the NMR signal intensity. The decrease in the signal intensity is inversely proportional to the distance (r^6) from the paramagnetic center (up to ~30-40 Å) (Fig. 1A).[1,2] Representative paramagnetic compounds for PRE are nitroxide spin radicals (e.g., *S*-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methanesulfonothioate (MTSL)),[3] transition metals (e.g., Mn^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+}, Cu^{2+} and Zn^{2+})[4] and several types of lanthanide ions (e.g., Gd^{3+}, Tb^{3+}, Dy^{3+}, Tm^{3+} and Yb^{3+}).[1]

Electrostatically polarized paramagnetic centers, for example when using lanthanide ions, show anisotropic magnetic susceptibility, which will cause pseudo-contact shift (PCS) of NMR-active nuclei in addition to the PRE effect due to self-alignment of the paramagnetic center against the static magnetic field (Fig. 1B).[5-7] PCS effects influence chemical shifts of the NMR-active nuclei depending on the distance between the paramagnetic center and NMR-observable nuclei, and on the angle between the static magnetic field and vector of the paramagnetic center-nuclei dipolar-dipolar interaction.[8] Furthermore, smaller distance dependency of PCS (r^3) than that of PRE (r^6) can provide longer distance information rather than the case of PRE. These indicate that PCS is a powerful methodology offering more accurate and precise information to determine relative orientations not only between the interacting protein-ligands but also the tertiary structure of the protein-ligand complex.

One simple way to generate paramagnetic effects is to covalently attach the paramagnetic compounds to the partner molecules.[9] PRE is able to observe minor population,[10-14] which is one of the excellent aspects of PRE. By introducing the PRE-inducible spin labeling onto the specific site of partner molecules, intermolecular orientation can be elucidated even when the population of the certain orientation is less than ~1%.[6,11] Although PCS is less sensitive to observe minor states of proteins compared with PRE,[15] it is also remarkably useful application as PCS provides angular information as described above. In addition, alignment of paramagnetic compounds against to the static magnetic field allows us to measure residual dipolar coupling (RDC) (refer to the next section) without using orienting media.[5]

Practical tips of PRE and PCS experiments - Attenuation of the signal intensity and alteration of chemical shift of ^{1}H-^{15}N or ^{1}H-^{13}C correlation signals of the NMR-visible protein are analysed in PRE and PCS experiments, respectively (Fig. 1A). The same experiments for references should be performed by using the diamagnetic sample whose physicochemical properties are identical to those of the paramagnetic sample, except for the absence of paramagnetism.[6] Paramagnetic substance should be immobilized on the solvent-exposed surfaces of one side of proteins.[9,16-19] Spin-label reagents, which have stable free radicals such as nitroxides (e.g., MTSL or 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO)), lanthanide metal ion-chelating chemicals, or ion-chelating peptide tags, are used as paramagnetic substances. These are chemically anchored or genetically fused to desired sites on the protein. In the case of calcium and magnesium binding proteins or metalloproteins, attachment of chemicals or tags above are not necessary as calcium, magnesium and metal binding centers of proteins can be utilized for immobilization of lanthanide ions.[20]

In many cases, surface-exposured side-chains on proteins, which do not sterically interfere interactions with partner molecules, are modified to attach paramagnetic compounds.[19] In general, free

sulfhydryl groups of the side chain of cysteine residues are widely used for a spin-labeling site on proteins as maleimide groups of spin-labeling reagents are covalently attached to the surface-exposed sulfhydryl groups through spontaneous formation of the stable disulfide bond.[19] If no cysteine residues are available in proteins of interest, a single cysteine residue is often artificially introduced to a desired site of the protein by performing site-directed mutagenesis.[19] In addition, if several intrinsic cysteine residues are available, they must be substituted with other amino acids (typically serine or threonine) except for the only one cysteine residue located in the intended site in order to attach only one paramagnetic compounds per one protein.

Meanwhile, lanthanide-binding peptide tag (LBT), which has one free cysteine residue in the N-terminus, is utilized for lanthanide ion attachment. Although one-point anchoring LBT is possible, a two-point anchoring method is more efficient way to immobilize lanthanide ions on the protein more firmly, and thereby suppressing fluctuations of the paramagnetic center.[18,21,22] This fluctuation-free LBT will induce stronger PCS and makes it possible to acquire more accurate angular information compared to the single-point anchored LBT experiment. LBT-fused proteins can be over-expressed and the sulfhydryl groups of the N-terminal cysteine residue of the LBT spontaneously form a disulfide bond with another cysteine residue on the same protein.[18]

RDC

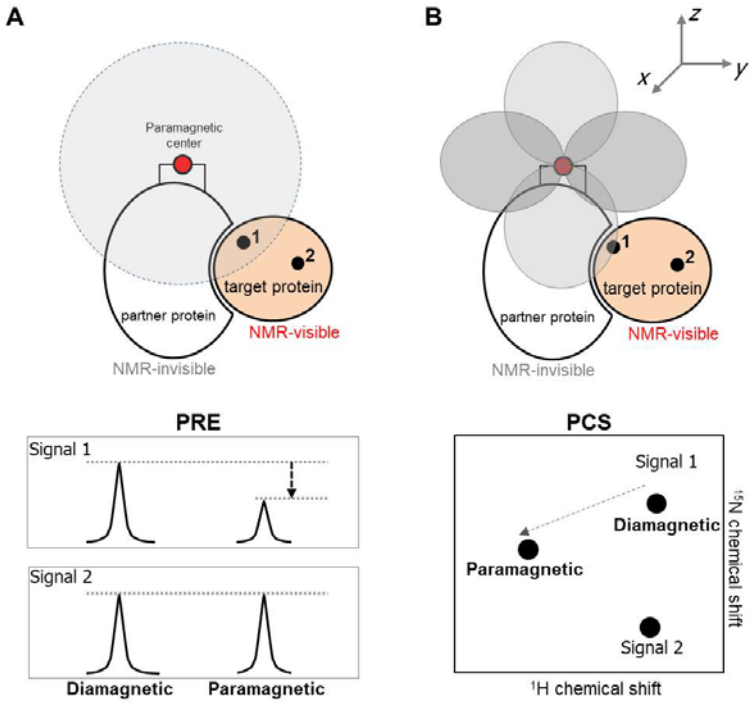

Figure 1. Illustration of paramagnetic effect-based NMR methods for investigation of protein-protein interactions. Effects of paramagnetic relaxation enhancement (PRE) (A) and pseudo-contact shift (PCS) (B) are shown. The alteration of the intensity (A) and chemical shift (B) of NMR signals of isotopically-labeled protein are analysed. PRE causes attenuation of the signal intensity depending on the distance, and PCS induces chemical shift changes depending on the distance and angle from the immobilized paramagnetic center (small red sphere). The region in which PRE and PCS, which affects the NMR signal, is highlighted with gray spheres with dotted line and four ellipses, respectively. Black spheres with numbering (in the target proteins) indicate the location of the residues, which are corresponding to the NMR spectra.

RDC - Dipolar-dipolar coupling between NMR-active nuclei, such as ^{1}H-^{15}N or ^{1}H-^{13}C, are averaged to zero in solution due to isotropic molecular tumbling.[23] However, when proteins are partially aligned in the static magnetic fields, the dipolar-dipolar coupling is not completely avaraged out as a result of the remaining anisotropic tumbling. Thus, the dipolar-dipolar coupling constant of the partially aligned proteins, so-called "residual" dipolar coupling (RDC), can be observed as summation of the *J*-coupling constant of the dipoles (Fig. 2). The RDC values provide valuable information on the angles between the dipole vector and the static magnetic field. Therefore, the orientation of proteins against alignment tensors in the static magnetic field is determined,[23] which is important to elucidate relative orientation of a protein relative to a partner molecule in a complex. Relative orientation between domains of multiple-domain proteins is also estimated,[24] which often plays a role in the modulation of protein function.

Preparation of RDC sample with orienting media - Orienting media, which can be aligned uniformly and spontaneously along the static magnetic field, are mixed with proteins of interest in an NMR sample solution. Then, proteins are partially aligned to the static magnetic field by transient physical collisions with the orienting media.[23] Depending on the physicochemical characters of the target protein, appropriate orienting media should be selected. Lipid bilayer discs (e.g. bicells) and ether/alcohol mixture (e.g. C12E5/hexanol system) provide liquid crystalline phases[23,25] and lamellar phases,[26] respectively. Filamentous bacteriophages (e.g. Pf1 phage)[27,28] and cylindrical polyacrylamide gels, in which the pore shaped gel is slightly stretched and elongated along the static magnetic field in an NMR sample tube by mechanical compression,[29] are also widely used. In order to achieve sufficient alignment of the target proteins, transient collisions between proteins and orienthing media should be occurred at a proper frequency. Therefore, optimal conditions to observe the signals of the target proteins with sufficient intensities, by suppressing non-specific interactions of proteins with orienting media, should be determined in advance of RDC experiments. By attaching paramagnetic ion, the protein also allows to be aligned spontaneously along the static magnetic field without orienting media.[5] In that case, preliminary optimization of sample solution is not necessary. In order to achieve proper collision between target protein and orienting media, several approaches have been applied. In the case of phage Pf1, for example, in which the surface is predominantly negatively charged, optimization of the concentration of the phage, the pH value (which should be >6 due to the stability of the Pf1 phage), and the salt concentration is essential to collect data with sufficient quality.[27] Furthermore, it is important to confirm stability of the orienting media in a designed solution prior to the initiation of RDC measurements. For example, it is useful to know that bicelles are relatively stable at lower temperatures than their phase transition temperature.[30,31]

Measurements and analyses of RDC - In order to correct the RDC with accuracy, optimization of the NMR parameters, such as the number of scans or time domain of each dimension, is also critical to obtain NMR data with sufficiently high signal-to-noise ratios. In general, RDC values are measured using IPAP-type or J-modurated HSQC spectra.[32,33] Comparison of the width between the split signals between aligned and reference (isotropic condition) samples reveals the RDC value (Fig. 2) as the splitting provides the summated values of the *J*-coupling constant and RDC from the aligned sample, but reference spectra provide only the *J*-coupling constant. The alignment tensors in a static magnetic field are calculated on the basis of the RDC value obtained using computer programs, such as PALES[34] or REDCAT.[35,36] Correction to NMR data with sufficiently high resolution and accurate peak picking are also important to estimate RDC values as precisely as possible since RDC values are generally as small as a few Hz-30 Hz.[37,38]

If the affinity between interacting proteins is weak ($K_d \gtrsim 1$ mM), it is difficult to obtain accurate RDC values in the complex state. Thus, the increase in the

concentration of proteins as high as possible (≳1 mM) may give an opportunity to obtain RDC values[14] although values will be weighted average values of the free and bound states in a fast-exchange regime as below:

$$D_{\text{bound}} = \frac{(D_{\text{obs}} - X_{\text{free}} \cdot D_{\text{free}})}{X_{\text{bound}}}$$

where D_{obs} is the observed RDC value in the presence of partner proteins. D_{free} and D_{bound} are the RDC values in the free and bound states, respectively. X_{free} and X_{bound} represent the fractions of the free and bound states of the target proteins, respectively.[39] D_{free} is directly measured in the absence of partner proteins. Consequently, the RDC value derived from the bound state is obtained if the population ratio of free and bound states is available by performing the analysis of chemical shift perturbation or other appropriate experiments. (4

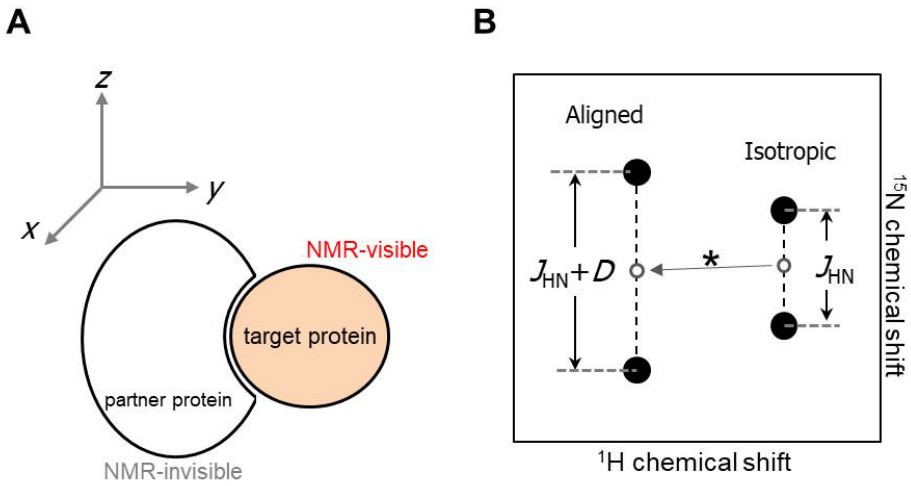

Figure 2. Schematic illustration of residual dipolar coupling (RDC) experiments. (A) Orientation of the dipole-dipole vector in the target protein in the cartesian space is determined. (B) Schematic representation of the two-dimensional ^{1}H-^{15}N spectrum for RDC. The "Aligned" and "Isotropic" (reference experiment) show peak splitting in the presence and absence of orienting media, respectively. J_{HN} and D indicate the constants of J-coupling and RDC, respectively. The asterisk indicates the chemical shift alteration in the midpoint of the split signals (denoted as small circles), which results from the PCS effect when the alignment is achieved by lanthanide ions.

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