NMR studies of protein-small molecule and protein-peptide interactions

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1 General introduction

1.1 Protein-ligand interactions

Protein-ligand interactions are essential for all biological processes. These interactions comprise biological recognition at the molecular level. When the ligand binds to the protein, it can regulate binding of other molecules, either by directly disrupting the interaction, competition of binding, or indirectly causing conformational changes. Therefore, understanding the detailed interactions between the binding partners is important to gain insight into the corresponding biological processes. This thesis focuses on two proteins: 12 kDa FK-506 binding protein (FKBP12) and plastocyanin (Pc). FKBP12 is used as a model protein for the development of a paramagnetic NMR-based methodology for studying general protein-small molecule interactions. The transient complexes formed by three different Pcs and small charged peptides, are created as models to elucidate the dynamic nature of encounter complexes and to provide fundamental understanding of transient protein-protein interactions.

FK-506 binding protein (FKBP)

FKBP is a protein family with *cis-trans* peptidyl-prolyl isomerase activity and belongs to the immunophilin family. FKBPs have been identified in many eukaryotes and function as protein folding chaperones for proteins containing proline residues. In this family, FKBP12 is a ubiquitous cytosolic protein implicated in several physiological processes. It is known in humans for binding the immunosuppressant molecules FK506 (tacrolimus)[1,2] and rapamycin (sirolimus),[3] which are used in treating patients after organ transplant and patients suffering from autoimmune disorders. The FKBP12-FK506 complex inhibits a serine/threonine phosphatase, calcineurin,[4] thus blocking the T-lymphocyte signal transduction pathway.[5,6]

The structures of FKBP12 in the free form, with ligands or other proteins, have been solved by NMR spectroscopy[7–14] and X-ray crystallography.[15–29] The overall structure is made up of five β-strands, an α-helix, and three loops.[15] The structure indicates a unique ligand binding

pocket, which can be subdivided into two sites.[30] Site 1 is the binding location for the pipecolinic acid ligands, whereas site 2 lies about 7 Å away. Selected ligands with a range of affinities for sites 1 or 2 have been reported, some of which have been linked to generate high-affinity ligands (Figure 1.1). This study pioneered the field of fragment-based drug discovery.

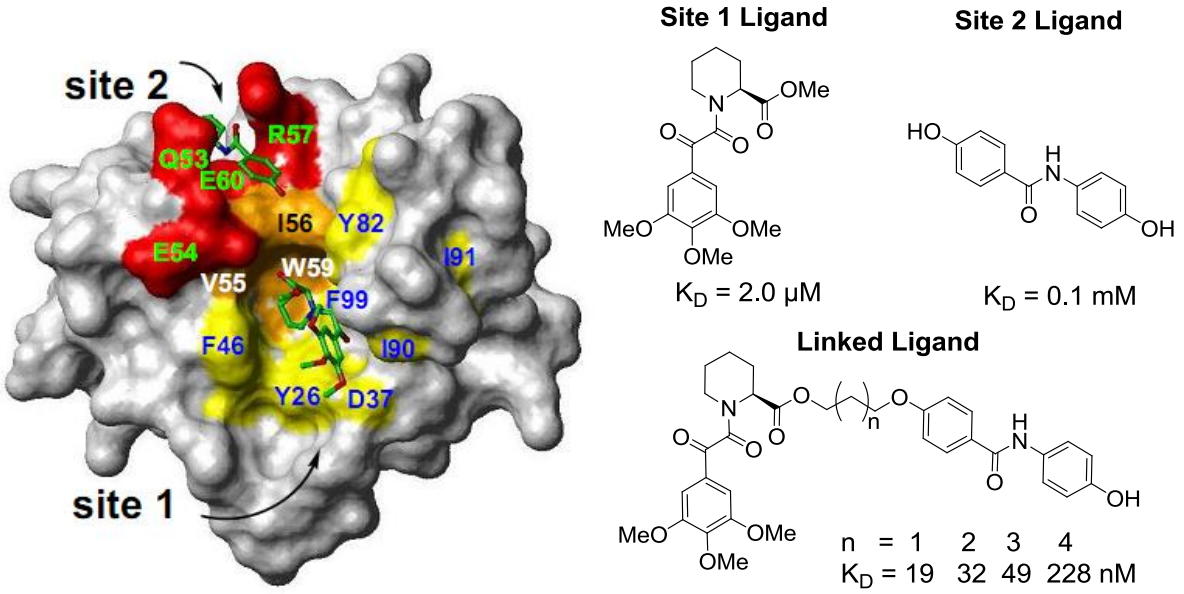

Figure 1.1: (Left) Surface representation of the two binding sites of FKBP12 with site-specific ligands. Residues in yellow and red are in site 1 and site 2, respectively. Residues in orange are shared between the two sites. For the ligands, atoms in red, blue and green are oxygens, nitrogens and carbons, respectively. (Right) Site-specific FKBP ligands are linked to produce high affinity ligands.[30]

Plastocyanin

Plastocyanin (Pc) is a blue copper protein which is involved in the electron transport process in photosynthesis. It transports electrons between cytochrome *f* of the b_6f complex and $P700^+$ of photosystem I (PSI) in plants, green algae and cyanobacteria.[31–34] Pcs from different species have been extensively studied and their structures are available from various plants and bacteria.[35–49] The overall structures of Pc are highly conserved from cyanobacteria to higher plants,[42] ranging from 97 to 105 amino acid residues, but large differences are present in terms of surface charge distributions. The typical structure of Pc consists of a β-sandwich consisting of seven β-strands and an irregular strand which contains a small helix in some Pcs. In poplar Pc, for example, the copper ion is chelated strongly by 3 residues, namely the S^{γ} of C84, the N^{δ} atoms of H37 and H87 and weakly with the S^{δ} of M92 (Figure 1.2).[50]

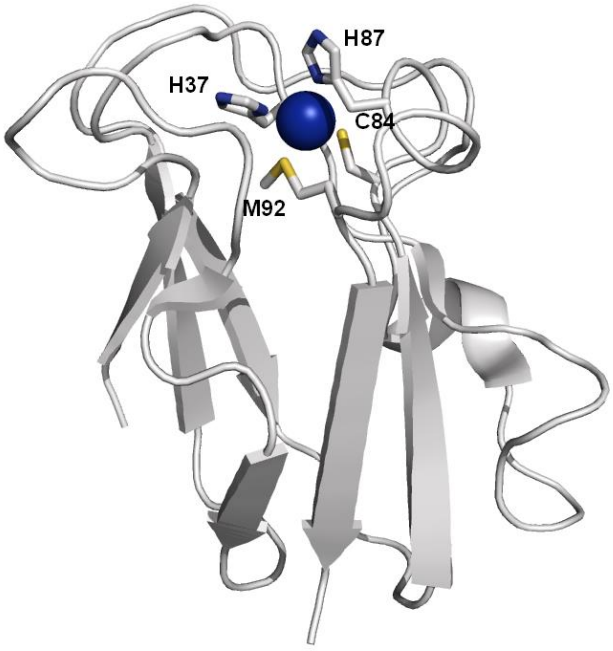

Figure 1.2: Structure of poplar Pc (PDB entry 1TKW).[47] Pc is shown as a grey ribbon, with the Cu ion as a blue sphere. The four residues (H37, C84, H87 and M92) that serve as the copper ligands are shown in sticks.

The function of Pc relies on two sites: a hydrophobic patch surrounding the solvent-accessible histidine copper ligand, and an electrostatically charged, remote surface area, whose nature varies from one organism to another. This charged area is mainly acidic in plants and green algae, whereas it ranges from acidic to basic in cyanobacteria. In higher plants, the hydrophobic patch surrounding the exposed His87 ligand and the distant acidic patch are potential binding sites for redox partners of Pc.[41,51–54] In the fern *Dryopteris crassirhizoma* Pc, on the contrary, the acidic region surrounds the hydrophobic patch.[43] Although the acidic patches are not conserved in bacteria, the hydrophobic patch is always present. The hydrophobic patches and the acidic patches play a key role in the dynamics of the complex.[51]

1.2 Fragment-based drug discovery

Fragment-based drug discovery (FBDD) is a powerful method for discovering high-affinity ligands for target proteins. The term "fragment" is used for small organic molecules with a molecular weight of <300 Da.[55] Fragment-based screening has several advantages over conventional high-throughput screening (HTS). First, due to the small size of fragments, the number of compounds that need to be screened is dramatically reduced to cover a greater chemical diversity space. Second, although fragment hits are weakly binding, they make high-quality interactions with the target to bind with sufficient affinity for detection, which has been interpreted as high ligand efficiency.[56]

In FBDD, detection of weak binding is of particularly interest, which leads to the development of various biophysical tools. Overview of various techniques applied in FBDD can be found elsewhere.[57–59] The following content describes three of the most commonly used techniques to characterize ligand binding. An overview of existing protein-ligand NMR approaches will be presented in Section 1.3.

X-ray crystallography

X-ray crystallography ins an information-rich technique that provides both hit validation and structural information in one step. It is often accomplished by analyzing protein crystals soaked in a cocktail of fragments.[60] This technique is a versatile tool to visualize the complex structures at the atomic level, although it is not possible to measure an affinity. However, many cases have shown failures in obtaining good quality crystals due to problems of ligand soaking, multiple binding modes, weak binding, or disruption of the crystal lattice caused by protein motions. It is also a huge investment of resource to obtain the simple yes/no answer in primary screening.

Surface plasmon resonance

Surface plasmon resonance (SPR) is a technique based on optical sensors. It provides binding measurements to characterize the kinetic properties of the biomolecular complex. In SPR, the target protein is immobilized on a sensor chip with a thin layer of gold on the surface, which is then exposed to the analyte in the flow channel.[61] Binding of the ligands results in changes of the refractive index on the surface of the sensor. This change is proportional to the number of bound molecules. The rate constants of ligand association (k_{on}) and ligand dissociation (k_{off}) are estimated by regression of the association and dissociation gradients at different ligand concentrations. The ratio of k_{off}/k_{on} is the binding constant (K_D). Therefore, SPR can quantitatively analyze the binding events.[62]

Isothermal titration calorimetry

Recently, thermodynamics involved in ligand binding has gained much interest in optimizing the structures of drug candidates.[63,64] Isothermal titration calorimetry (ITC) is one of the few techniques which measure both the binding affinity and the thermodynamic terms that contribute to the affinity: the binding enthalpy (ΔH) and the binding entropy (ΔS). It has no requirement for chemical modification, labeling, immobilization or limit on the size of interacting species. The binding curve allows extraction of the thermodynamic parameters, the stoichiometry and K_D. This technique has been used to provide a comparison of energy terms in the development of complete series of drugs.[63] To achieve high affinity, both ΔH and ΔS are required to contribute favorably to binding. Therefore, the thermodynamic data can provide valuable information to complement existing tools to facilitate lead discovery and optimization.[64,65]

1.3 NMR techniques for protein-ligand interactions

NMR has been recognized as a powerful tool to characterize macromolecular structures and dynamics. It is also utilized extensively in screening ligand binding to protein targets in the early stage of fragment-based drug discovery.[66–70] One of its key advantages is that it can detect and quantify weak interactions (K_D μM-mM) without prior knowledge of protein structures. In addition, NMR can be applied to obtain structural information for both the protein target and the ligand with atomic resolution for subsequent optimization of weak binding hits into high affinity drug candidates.

Here, a summary of NMR techniques used for protein-ligand interactions is presented. They are subdivided into ligand- and protein-observed techniques. In the protein-observed techniques, the influences of ligand binding on the spectra of the target are detected, whereas in the ligand-observed techniques, changes induced in the ligand's NMR resonances upon binding are observed. Recently, a variety of techniques using paramagnetic NMR and combining NMR and computational approaches are gaining interests. These techniques are also described here.

Protein-observed NMR

Binding of ligands to targets leads to changes in the chemical shifts, which are most conveniently monitored in mixtures of ligands with a ^{15}N- or ^{13}C-labeled protein. Through a series of target 2D-HSQC spectra with different ligand concentrations, the dissociation constant K_D can be derived. If the assignments of the target are available, the location of the binding site may be easily derived. This strategy is called chemical shift perturbation (CSP), also known as SAR (Structure-Activity Relationship) by NMR,[30] chemical shift mapping or complexation-induced change in chemical shift (CIS), and has been successful applied in numerous cases for screening and optimizing lead compounds. However, HSQC-based methods are presently limited to macromolecular complexes with a molecular weight below 30 or 100 kDa (in combination with TROSY).[71,72] Another limitation is the requirement for large amounts of isotopically labelled protein, which can only be obtained if an effective expression system in an isotope-labelled medium is available. Despite this, side-chain specific isotope labelling has significantly increased the scope of protein systems that are amenable for SAR by NMR,[73,74] and the cost-effective ^{13}C labelling has made screening of 100,000 compounds practical.[75] To obtain information of ligand binding at atomic resolution, intermolecular NOEs can be observed using isotope filtered/edited NOESY experiments.

This works best for tight-binding ligands. Transferred NOE experiments can be used for weak binding ligands.

Ligand-observed NMR

Another category of techniques is based on the changes in the ligand NMR signals in the presence of the target. These methods are often used for proteins with Mw 15–100 kDa. Given that the ligand is in fast exchange on the NMR time scale, the observed signals are the weighted average of the free and bound ligand. Most techniques used for primary screening detect a response in the form of a reduction (disappearance) or increase (appearance) of signal amplitude due to changes in relaxation rates or magnetization transfer. Competition/displacement experiments can also provide information of the binding site and the relative affinity if the location and affinity of the first ligand is known. Paramagnetic labelling on the ligand or the protein can also be used to obtain information of the ligand binding by monitoring changes in the relaxation or chemical shifts of nuclei located at different regions of the molecule. An overview of the techniques is shown in Table 1.1.

Saturation transfer difference (STD) was initially designed to identify small-molecule ligands that bind to a receptor protein from a mixture of compounds.[76] The spectrum of the receptor is saturated by selective irradiation that does not influence the ligand resonances. Spin diffusion in the receptor leads to saturation of its spectrum and causes saturation transfer by NOE to the bound ligand. If the ligand is in fast exchange, signal reduction of the free ligand can be detected. This method has also been applied to identify the ligand binding epitope[77] and the structure of a bound ligand.[78,79]

With the combination of STD and highly deuterated proteins with selectively protonated amino acids (Ile, Leu, Val, and Met), one can also identify ligand binding sites. This is used in SOS-NMR (*s*tructural information using *O*verhauser effects and *s*elective labelling).[80] It requires an unlabeled protein as the positive control and a perdeuterated protein as the negative control.

WaterLOGSY (water-ligand observed via gradient spectroscopy)[81–83] is based on the intermolecular magnetization transfer and spin diffusion of the excited bulk water. Small molecules that interact with the ‘bound’ water on the protein (binders) behave differently from those that interact with the bulk water (non-binders), resulting in different signs in NMR signals. WaterLOGSY has been widely applied in primary screening.

Target immobilized NMR screening (TINS) uses a solid support to immobilize the target and therefore slows down the tumbling rate of the immobilized target, causing much faster

relaxation relative to the free molecules in the solution phase. TINS uses difference in the spectrum of the fragments in the presence of the target to detect binding, and of a reference protein to cancel out non-specific binding. It was first demonstrated with FKBP12,[84] and has been successfully applied on membrane proteins DsbB[85] and adenosine A_{2A} receptor.[86,87]

INPHARMA (*i*nterligand *N*OEs for *phar*macophore *ma*pping)[88] detects protein-mediated ligand-ligand interactions in competition binding. The restraints come from protein-mediated inter-ligand nuclear Overhauser effects (NOEs) observed between two competitive ligands. If the structure of the ligand–protein complex is known for one ligand, the complex structure for the other ligand can be estimated based on the intermolecular NOEs.

Paramagnetic NMR

Paramagnetic effects originate from unpaired electrons, which can affect the magnetic properties of nuclei in the vicinity. The theory of paramagnetic effects is explained in Section 1.4. These long-range effects are strongly distance and, in some cases, orientation dependent, and are subsequently observable in NMR spectra as pseudocontact shifts (PCS), paramagnetic relaxation enhancement (PRE) and/or residual dipolar coupling (RDC).[89–91] Therefore, paramagnetic NMR can provide valuable information for elucidation of protein structure, conformational dynamics, protein-protein interactions and protein-ligand interactions. Here the existing paramagnetic NMR-based approaches for protein-ligand complexes are described.

SLAPSTIC (*s*pin *l*abels *a*ttached to *p*rotein *s*ide chains as a *t*ool to identify *i*nteracting *c*ompounds) uses spin-labels, such as 2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPO), attached on the lysine residues of the target protein to detect ligand binding from a mixture of compounds.[92] The bound ligand shows signal reduction in $T_{1\rho}$ relaxation experiments. This approach can be used for primary screening. A similar approach has been applied in screening a second ligand that binds close to an already identified primary ligand attached with a TEMPO spin label.[93] Such approaches have been demonstrated to find inhibitors for Bcl-x_L[93] and Bcr-Abl kinase.[94] The use of paramagnetic ions instead of spin labels for ligand screening has also been demonstrated by substitution of diamagnetic Zn^{2+} with paramagnetic Co^{2+} in the matrix metalloproteinase MMP-12.[95]

To further determine the structure of the protein-ligand complex using paramagnetic effects, several studies have appeared in recent years. A combination of PCS and RDC with respect to a single fusion C-terminus lanthanide-binding peptide tag (LBT) was applied to assist structure determination of a carbohydrate-protein complex.[96] Later, the use of PCS was

applied to determine the pose of a ligand bound to a metalloprotein.[97] This requires an enzyme with a native metal chelating site near the binding pocket, and the use of very strong lanthanides. PRE assisted ligand docking with a spin-label peptide bound specifically to a protein was also reported.[98] Recently, a two-point anchored N-terminus lanthanide binding peptide (LBP) was applied for both ligand screening and determination of protein-peptide complexes.[99] In this approach, ligand screening was achieved by observing paramagnetic line broadening for both the target protein and its bound ligand in 1D-^{1}H NMR, and the ligand binding modes were derived by paramagnetic pseudocontact shifts (PCS).

Combination of NMR and computational approaches

The advantages of computation over experimental approaches are the fast speed and the possibility of studying challenging complexes that are difficult to study experimentally. However, computational approaches do not always provide accurate prediction. By integrating the experimental information, computational studies can provide more valuable biological insight. Various approaches combining NMR data and computation have been developed to extract the information of ligand binding.[100–102]

Typically, if no significant conformational changes occurs on the protein upon ligand binding, the ring current effect generated by aromatic rings in ligands is the most important factor of the observed CSPs on the protein.[103] Detailed calculations of ligand-induced CSPs were demonstrated useful to determine ligand binding modes.[104–106] However, the quantitative CSP methods are limited to aromatic ligands, due to the requirement of ring current shifts produced by the ligand.[104] Qualitative CSPs can also be used to both guide and filter the calculations, reducing the grid space and minimized energy in the process.[107]

Protein-ligand NOE matching approach[108] utilizes data from 3D ^{13}C-edited, ^{13}C/ ^{15}N-filtered HSQC-NOESY spectra for evaluating ligand binding poses. Only the ^{1}H NMR assignments of the bound ligand are essential. No protein assignments are required but can be used if the information is available. Predicted filtered NMR spectra are used and, in combination of ligand pose evaluation, refined to match with the experimental spectra. This method required a single protein sample which has to be ^{13}C- and ^{15}N-labelled.

Table 1.1: Overview of NMR techniques for studying protein-ligand interactions

Type	Technique	Principles and applications	Limitation
Magnetization transfer	STD	Primary screening and epitope mapping.	Large protein (>10 kDa) preferred.
	waterLOGSY	Primary screening; good for very hydrophilic targets and ligands.	Large protein (>10 kDa) preferred. Labile protons can give false positives.
	SOS-NMR	STD-based; observe differences between protonated and deuterated proteins	Requires selective amino acid labeling.
	INPHARMA	NOE-based, detect inter-ligand NOEs; active site and bound ligand conformation.	Observation depends on kinetics and ratio of ligand affinities.
Relaxation/ Paramagnetic NMR	TINS	Bound ligand has enhanced R_2 due to immobilization on the resin; primary screening; applicable to membrane proteins.	Not applicable to tight-binding ligands.
	SLAPSTIC	Attach TEMPO on lysine residues; detect line-broadening of bound ligand by $T_{1\rho}$; primary screening.	Results can be ambiguous if the lysine position is unknown.
	Lanthanide binding peptides (LBP)	LBP co-expressed with the protein; screening and binding pose information.	^{15}N-labeled proteins required to determine tensor parameters.
NMR driven Computation	CSP guided docking	CSP-based; quantitatively calculate ring current induced shifts from the ligand or qualitatively guide docking; ligand binding mode.	Quantitative methods limited to aromatic ligands. ^{13}C/^{15}N-labeled proteins required.
	Protein-ligand NOE matching	NOE-based; only need ligand assignments; protein assignments not essential; ligand binding mode.	^{13}C/^{15}N-labeled proteins required.

1.4 Theory of paramagnetic effects

Due to its isotropic and anisotropic effects, paramagnetic NMR can be a versatile tool to extract position-specific distance and angular information with respect to the paramagnetic center. Subsequently, the information can be used to determine and refine the structures of protein-ligand and protein-protein complexes, facilitate resonance assignments, and to study the sparsely-populated states and the dynamics.[89,90,109–112] The three most commonly used paramagnetic effects are paramagnetic relaxation enhancement (PRE), pseudocontact shift (PCS) and residual dipolar coupling (RDC). The principles of paramagnetic effects applied in this thesis, PRE and PCS, are explained below. Detailed theories of paramagnetic effects, including RDC, can be found in literature reviews and books.[89,113–119]

Paramagnetic relaxation enhancement (PRE)

Paramagnetic centers invariably cause line broadening in the NMR spectrum owing to paramagnetic enhancements of the transverse relaxation rate, R_2. This effect is called paramagnetic relaxation enhancement (PRE). It shows a distance dependency of r^{-6}. The relation between the nucleus-to-electron distance (r_{IM}) and relaxation rate enhancement (R_2^{para}) is shown in its simplified form in Equation 1.1:

$$R_2^{para} = \frac{1}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma_I^2 g_e^2 \mu_B^2 S(S+1)}{r_{IM}^6}\left(4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2}\right) \tag{1.1}$$

Where μ_0 is the permeability of free space, g_e is the electronic g-factor, μ_B is the Bohr magneton, S is the total electron spin quantum number, γ_I is the proton gyromagnetic ratio, $\omega_I/2\pi$ is the Larmor frequency of the proton, and τ_c is the correlation time.

R_2^{para} can be retrieved by comparing the peak intensities in the paramagnetic and diamagnetic forms (Equation 1.2):

$$\frac{I_{para}}{I_{dia}} = \frac{R_2^{dia}\exp\left(-R_2^{para}t\right)}{R_2^{dia}+R_2^{para}} \tag{1.2}$$

where I_{para} and I_{dia} are the peak heights under paramagnetic and diamagnetic conditions, respectively, and t is the INEPT time in a HSQC experiment, the time during which transverse relaxation was active.

PREs fall off with the sixth power of the distance, yielding a limited useful distance range. On the other hand, they offer the possibility to study minor conformational species of molecules and transient complexes in solution.[120,121]

Pseudocontact shifts (PCS)

Pseudocontact shifts (PCS) are defined as the difference in chemical shifts between the paramagnetic and diamagnetic states. The PCS gives information on the distance and angle between a nucleus and the paramagnetic center according to Equation 1.3:[113]

$$\Delta\delta^{PCS} = \frac{1}{12\pi r^3}\left[\Delta\chi_{ax}\left(3\cos^2\theta - 1\right) + \frac{3}{2}\Delta\chi_{rh}\sin^2\theta\cos 2\varphi\right] \tag{1.3}$$

where r, θ, and φ are the polar coordinates of the nucleus with respect to the principle axes of the χ-tensor and $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$ are the axial and rhombic components of the χ-tensor, respectively. The r^{-3} distance dependency is much weaker than the r^{-6} distance dependency of PRE. PCS can therefore be measured for nuclear spins that are far from the paramagnetic center, not strongly affected by PRE. Figure 1.3 depicts the characteristics of PRE and PCS.[90,111]

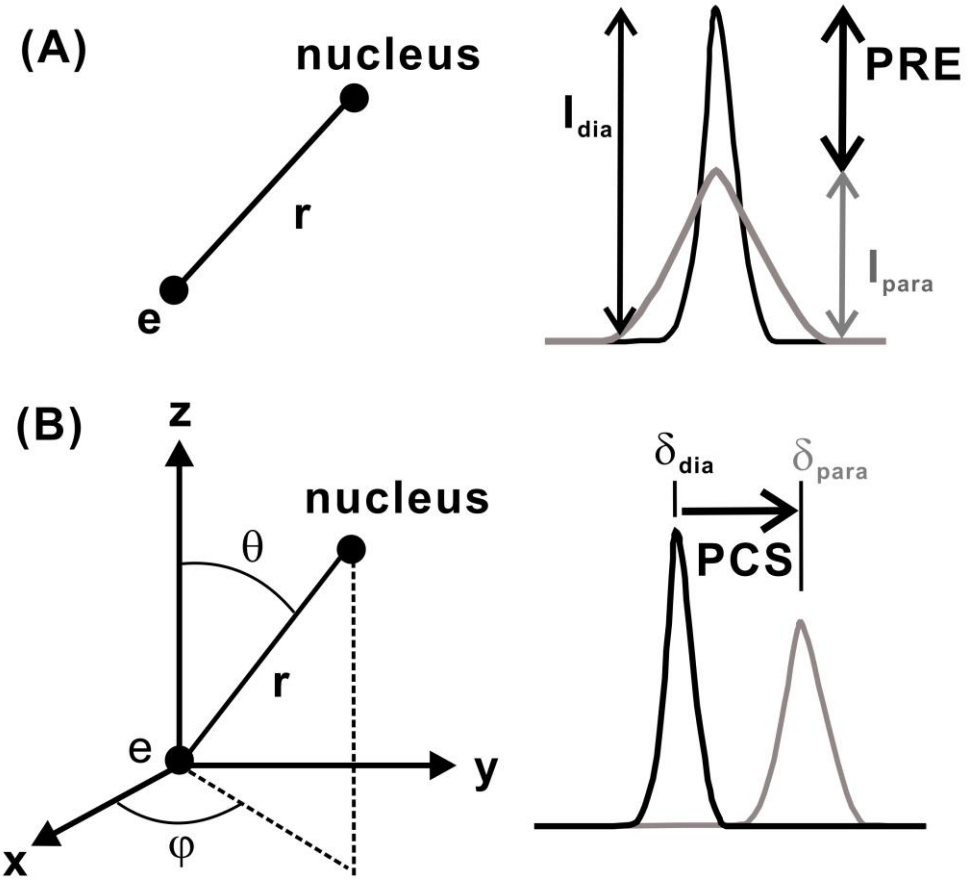

Figure 1.3: Principles of PRE and PCS. (A) PRE depends on the distance between electron and nuclear spin, and is observed as intensity difference between the paramagnetic and diamagnetic states. (B) PCS is observed as chemical shift differences between the paramagnetic and diamagnetic states. For PRE and PCS the intensity ratios or chemical shift differences of NMR resonances between a paramagnetic vs. a diamagnetic protein are used for structural studies.

1.5 Generation of paramagnetic effects on biomolecules

The use of paramagnetic effects in metalloproteins has been pioneered by Bertini *et.al.*[114,116] Later it was expanded from metalloproteins to non-metalloproteins by external paramagnetic tags. Common approaches to introduce paramagnetic effects on the target protein are presented:

Direct metal substitution

Lanthanides (Ln) do not exist in natural biological systems. For metalloproteins containing calcium, magnesium and manganese, the similarity between trivalent lanthanides (Ln^{3+}) and Ca^{2+} in terms of ionic radius and oxophilicity allows direct metal substitution, providing a valuable tool for structural dynamic studies.[122–127] Cu^{2+}- and Fe^{3+}-binding proteins were also used for obtaining paramagnetic restraints without substituting the metal ions.[128,129]

Metal-chelating tags

In recent years, a variety of site-directed lanthanide-binding tags (LBTs), spin-label compounds and lanthanide binding peptides (LBPs) have been developed to provide great opportunities for applying paramagnetic NMR on non-metallic proteins.

LBPs that mimic metal binding sites of metalloproteins can be inserted by standard molecular biology techniques, at either terminus or in the loop regions of non-metalloproteins, to serve as paramagnetic center once they are chelated to the appropriate metal.[130–135] This type of peptides can also be attached to the protein via cysteine modification.[136]

Another way to incorporate Ln is to use organic compounds as LBTs. The attachment of LBTs through cysteine thiol modifications is advantageous since it affords a rational means of generating Ln-tagged proteins with desired orientations between the Ln ion and the target proteins for NMR application.[133] However, site-specific incorporation of cysteines and removal of other solvent-accessible cysteines can sometimes results in problems in folding and purification. These problems can be eliminated by using metal-binding unnatural amino acids.[137] The amino acids can be incorporated site-specifically by a modified expression system.[138] Despite its relatively weaker binding affinity to lanthanides compared with other LBTs and LBPs, this approach has no limitation on the amino acid sequence, and therefore provides more possibilities in expanding NMR structural biology.[139]

Spin labels

Nitroxide spin labels are the simplest and most widely used paramagnetic tags for PRE.[89] These nitroxides contain an unpaired electron in a sterically protected environment, making them stable radicals under physiological conditions.[140] In most cases, they are usually attached to a surface cysteine residue, and therefore are mobile groups. This has to be taken into account when deriving PRE restraints.[141] Recently, an amino acid with a stable radical and a rigid conformation, 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC) has been introduced into peptides by using conventional solid phase synthesis.[142] Due to its relatively bulky structure, placing TOAC outside the recognition motif is preferable to minimize the effect on binding and to extract PRE information.[143]

1.6 Thesis outline

The aim of this thesis is to study protein-small molecule and protein-peptide interactions by various NMR approaches, including NOE, PCS and PRE. Particular interests are emphasized on paramagnetic NMR in the following topics: (1) implementation of a general methodology of paramagnetic NMR to elucidate ligand binding modes; and (2) dynamics of transient protein-peptide encounter complexes.

Chapter 2 describes the initial ligand studies for FKBP12. In order to characterize the behavior of the ligands and the chemical shift perturbations on the protein target, a set of small-molecule ligands selected from a TINS competition screen and known FKBP12 ligands from literature were studied by 1D-^{1}H and [^{1}H, ^{15}N]-HSQC NMR. It was found that the chemical structure and the binding affinity of the ligand could have dramatic effects on their behavior in the 1D-^{1}H diamagnetic and paramagnetic NMR spectra.

Chapter 3 discusses the NOE-based structure of a small-molecule ligand bound to FKBP12. Multi-dimensional NMR was applied to complete the assignments of the protein in the free and bound form with the ligand. The ligand was first identified in TINS screening and later validated with chemical shift perturbations (CSPs). However, CSPs alone were not sufficient to elaborate the binding site, as perturbations were found in both site 1 and site 2 of FKBP12. Therefore, the actual binding site was determined by intermolecular NOE restraints from isotope filtered/edited NOESY experiments. Despite possible dynamics present in both the ligand and the protein, the site is in agreement with the binding sites identified for known FKBP12 ligands.

In Chapter 4, the same protein-small molecule structure was determined independently by a paramagnetic NMR approach, mainly using ligand PCS. The paramagnetic effect was implemented by using a lanthanide-binding tag, CLaNP-5.[144] The structural calculations were carried out in two individual ways: with either the predicted or the optimized tensors. A comparison between these two PCS-derived structures and the NOE-derived structure is presented. The advantage and disadvantage of this methodology compared with other NMR approaches is addressed.

In Chapter 5, the interactions between tetralysine peptides and plastocyanins from different species – poplar tree (*Populus nigra*), Japanese fern (*Dryopteris crassirhizoma*), and a cyanobacterium (*Phormidium laminosum*) – were investigated by PRE NMR and theoretical Monte Carlo simulations. Tetralysine peptides have been shown to interrupt the binding between Pc and cytochrome *f* and to cause subtle structural changes on the copper site of Pc.[44,145–147] In this study, lysine peptides that contain TOAC spin-labels[143] were applied to investigate the dynamics of the transient Pc-peptide complexes.

Finally, in the concluding remarks in Chapter 6, the results of these studies are summarized and integrated.