Article

NMR spectroscopy – a simple yet powerful tool in chemical biology

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Introduction

Molecular recognition events between proteins and ligands play essential roles in almost all biological processes in cells, ranging from transport to signalling, metabolism to biosynthesis. The ligands involved in these biological processes can be small molecules, peptides, proteins, metal ions, nucleic acids or other biomolecules. Studies of these binding events at the molecular and atomic levels can therefore lead to deeper understandings of their corresponding biological processes. In addition, modern drug discovery programmes often involve the development of small molecule compounds that bind specifically to a target protein receptor.[1–3] The ability to measure, monitor, study and characterise protein-ligand interactions and complexes are therefore of paramount importance in the fields of chemical biology, medicinal chemistry and drug discovery.

Amongst the many biophysical methods that are available to study protein-ligand interactions,[4,5] nuclear magnetic resonance (NMR) is unique as it can provide information about almost all aspects of protein-ligand interactions. It has found diverse applications including ligand screenings, binding constant (K_D) measurements, structure and conformation determinations, and dynamics studies of both proteins and ligands.[6,7] Many NMR experiments are available, which can be broadly divided into two approaches. One approach is called "ligand-observed NMR", which involves the observation of ligand resonances. The other approach is called "protein NMR", which involves the monitoring of isotopically labelled protein resonances.

A number of excellent reviews have already been published focusing on the technical and practical aspects of both ligand-observed and protein-observed NMR experiments.[8-19] The aim of this article is to provide researchers, especially those who work at the interface between chemistry and biology but who may not necessarily be familiar with NMR, overview descriptions of some of the most popular NMR techniques in the studies of protein-ligand interactions.

Ligand-observed NMR

Ligand-observed NMR methods are widely used for the studies of protein-ligand interactions in both academia and industry. For example, it is used extensively as a screening tool in the area of fragment-based drug discovery.[20–23] There are several reasons for the popularity of ligand-observed experiments (as opposed to protein NMR). Firstly, they do not require isotopically labelled protein. Secondly, it is possible to screen mixtures of compounds in a single experiment provided there are no overlaps in chemical shifts. Thirdly, the applicability of ligand-based NMR experiments is not limited by the size of the protein.

There are three main types of ligand-observed NMR experiments. One involves observing changes of the ligand resonances in the presence and absence of a protein receptor. Another involves the use of a reporter ligand, which is in competition with the ligand-of-interest for the same binding site. A third type relies on the nuclear Overhauser effect (NOE). It involves monitoring the signals of the free ligand that is in exchange with the bound ligand.

Direct ligand observation

Direct ligand observation is the simplest form of ligand-observed NMR experiments (Fig. 1a). Ligand binding can be indicated by comparing changes in the NMR parameters of the ligand in the absence and presence of the receptor protein. When a ligand molecule is bound to a protein, it experiences different chemical environments and rotational correlation times. The observed NMR parameters will therefore reflect the population weighted average of the free and bound forms of the ligand provided the exchange between the two forms is fast. Binding constants (K_D) can also be obtained by following changes in the ligand NMR parameters at different protein or ligand concentrations through titration experiments.[24,25]

Any measurable NMR parameters may be monitored. Changes in chemical shift, linewidth and peak intensity are the most common as they can be readily followed using simple 1D spectra. The differences in ligand linewidth and peak intensity can be further enhanced by using relaxation-edited experiments such as the Carr-Purcell-Meiboom-Gill (CPMG) sequence by exploiting the differences in longitudinal (T_1) and transverse (T_2) relaxation time constants between the ligand free and protein-bound forms.[26] The most common nuclei for direct observation are ^{1}H [27,28] and ^{19}F [28–31] although other spin ½ nuclei including ^{13}C,[29] ^{15}N [32] and ^{31}P [33] have also been used. Nuclei with a large chemical shift range (such as ^{19}F and ^{31}P) have an additional advantage because they possess large chemical shift anisotropy (CSA). In such cases, even weak binding interactions may lead to line broadening in the ^{19}F or ^{31}P spectra owing to the strong T_2 dependent line broadening pathway.[33,34]

Generally, for a fast exchange system, a slight excess of ligand (usually around 10 fold over protein concentration) is used in order to increase the population of the protein-ligand complex. Whilst this is optimal for medium-affin-

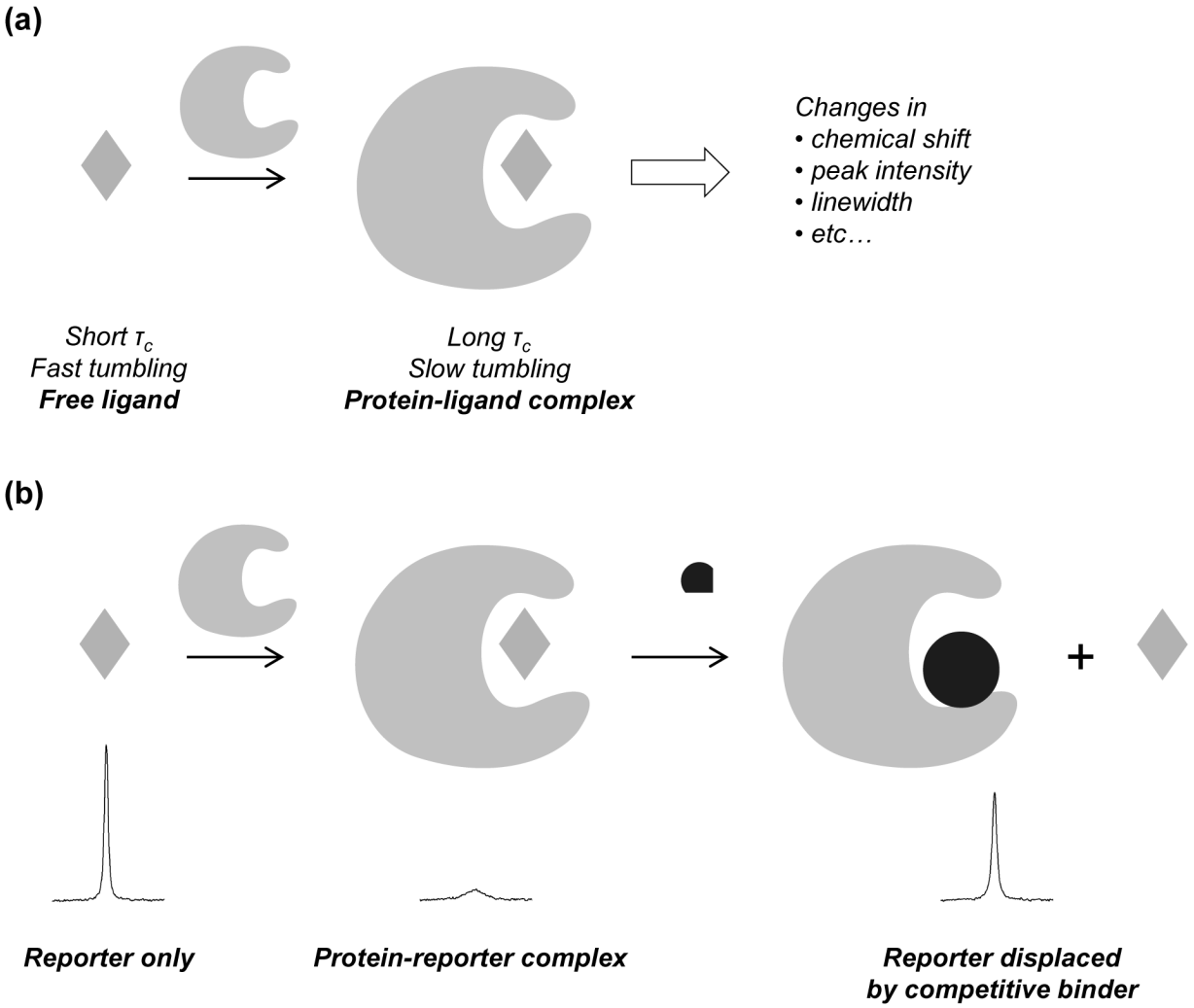

Fig. 1. (a) Scheme illustrating the principle of direct ligand observation in NMR screening. (b) Scheme illustrating the principle of reporter ligand displacement method in NMR screening.

ity protein-ligand systems, this setup may lead to false negatives for high-affinity ligands. This is because the dissociation rate (k_{off}) of high-affinity ligands from their protein-ligand complexes is usually slow. Under a slow exchange regime, the observed NMR signals no longer reflect the weighted populations of the free ligand and protein-ligand complex, and instead, they only reflect the free ligand population. Therefore, it may be difficult to detect slight decreases in peak intensity (as a result of ligand binding) as the spectrum is dominated by a large excess of free ligands. In such cases, a ~1:1 ratio of protein to ligand concentration can be used.

A recent example of applying direct ligand observation in ligand screening is reported by Manzenrieder *et al*. (Fig. 2).[33] As a proof-of-principle experiment, a library of five phosphorylated compounds was screened against the protein thermolysin, and binding was monitored by proton decoupled ^{31}P ($^{31}P\{^{1}H\}$) experiments. Upon addition of the protein, the ^{31}P signal of one of the five compounds disappeared, indicating binding of that particular compound to thermolysin. In order to test the binding specificity, a high-affinity known binder was then added to the mixture, which led to the reappearance of the vanished signal. This indicates the two compounds were competing for the same binding site. The authors also tested the lower limit of protein concentration that is required to detect binding. At 500 μM ligand concentration, they found that binding can be observed with as little as 3 μM protein owing to the strong CSA effect of ^{31}P.

Reporter displacement method

The reporter displacement method is an extension of the direct ligand observation method. It is a competition-based experiment, in which changes in the NMR parameters of a reporter ligand in the presence and absence of the ligand-of-interest are being monitored (Fig. 1b).[33,35–40]

There are two prerequisites for this method. Firstly, the ligand-of-interest and the reporter should compete for the same binding site, which otherwise may lead to false negative results. Secondly, the availability of a good reporter ligand is crucial: the k_{off} of the reporter should be sufficiently fast, and there should be no chemical shift overlap between the reporter and the ligands-of-interest. Binding constant of the ligands-of-interest can also be obtained by following the recovery of the reporter signal at different ligand concentrations, provided the K_D of the reporter to the target protein is known.

There are several advantages of using a reporter to follow ligand binding over direct ligand observation. Firstly, it

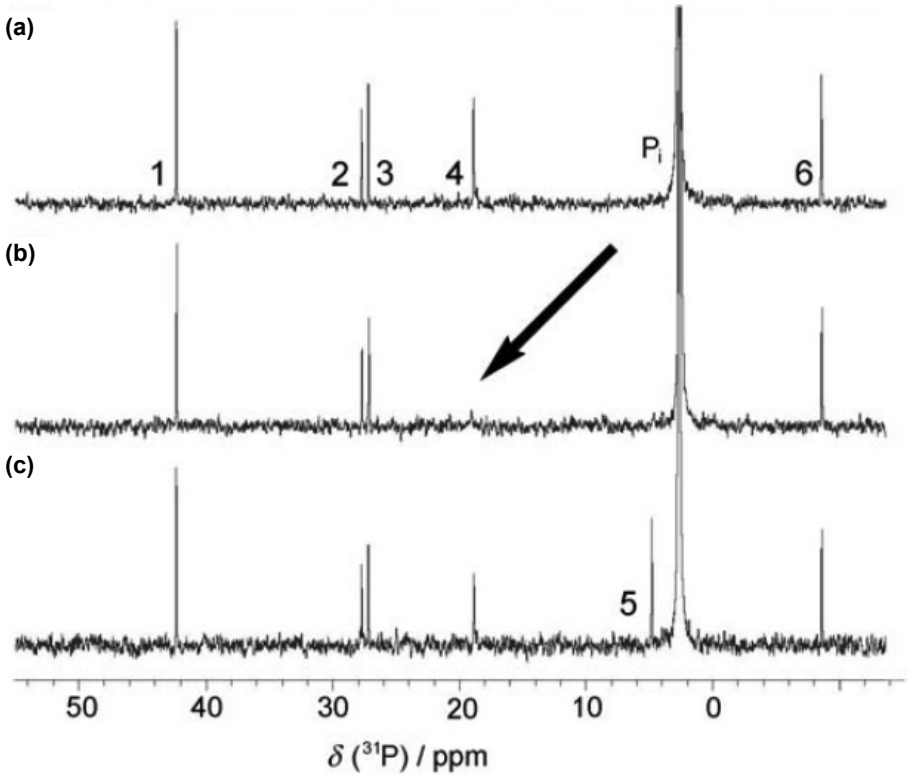

Fig. 2. Manzenrieder *et al*. applied $^{31}P\{^{1}H\}$ NMR to screen a mixture of five phosphorylated compounds to the protein thermolysin. (a) Library of five phosphorylated compounds (**1**, **2**, **3**, **4**, **6**; 0.5 mM each). (b) The signal of compound **4** disappeared upon addition of 0.25 mM thermolysin. (c) Addition of 0.5 mM compound **5**, a high-affinity binder of thermolysin, led to the recovery of the signal of compound **4**. Reprinted with permission from *Angew. Chem. Int. Ed.* **2008**, *47*, 2608–2611. Copyright 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

provides site-specific binding information and therefore false positives due to non-specific binding can be minimised. Secondly, this method can be used to detect high-affinity ligands. This is because the experiment does not rely on the exchange between free and protein-bound forms of the ligand-of-interest, therefore it does not suffer from false negative results arising from slow ligand k_{off} as in the case of direct ligand observation.

We have recently applied the reporter displacement method to study ligand binding to prolyl hydroxylase domain 2 (PHD2), an enzyme that is involved in human oxygen sensing (Fig. 3).[38] 2-Oxoglutarate (2OG), the co-substrate of the enzyme, was used as the reporter ligand. As most PHD2 inhibitors to date are designed as 2OG competitor, binders can be readily identified and ranked according to their binding strengths. The simplicity of this method allows it to be applied routinely to screen and quantify novel binders for PHD2 and other oxygenases that utilise 2OG as a cosubstrate.

Nuclear Overhauser effect (NOE)-based methods

Another approach to study protein-ligand interactions is to utilise the nuclear Overhauser effect (NOE). Several techniques were developed based on this concept, the two most common techniques are saturation transfer difference NMR (STD-NMR)[41] and the water-ligand observation with gradient spectroscopy (waterLOGSY) method.[42]

STD-NMR

The STD-NMR method is based on the NOE between proteins and ligands (Fig. 4).[41] It is a combination of two experiments. In the first "on-resonance" experiment, radiofrequency pulses are applied to selected protein resonances for a defined length of time. Upon irradiation, all protein resonances become rapidly saturated by spin-diffusion. Magnetisation can also spread by the same process in an intermolecular fashion onto the bound ligands, which will lead to (partial) saturation of ligand resonances. If the ligand dissociates sufficiently fast, the saturation can be detected as a reduction in signal intensity in the spectrum. An "off-resonance" reference spectrum, which is essentially a normal ^{1}H spectrum, is also recorded without protein saturation in otherwise the same experimental conditions. Subtraction of the two spectra will lead to a "difference spectrum" in which only signals experiencing saturation are visible.

It is important to select a region of the protein that is far away from any ligand resonances for the selective on-resonance irradiation of the protein in order to avoid false positive results. The amount of time for the selective irradiation (saturation time) is typically between 1 and 5 seconds. A ligand excess of around 100 fold is usually used to ensure all the proteins are saturated with ligand molecules.

STD effects can be quantified by the STD factor, which is the fractional saturation of the on-resonance spectrum relative to the off-resonance spectrum. When comparing samples of different ligand excess, the STD amplification factor can be used. The STD amplification factor is the multiple of the STD factor and ligand excess. A mathematical representation is given below:

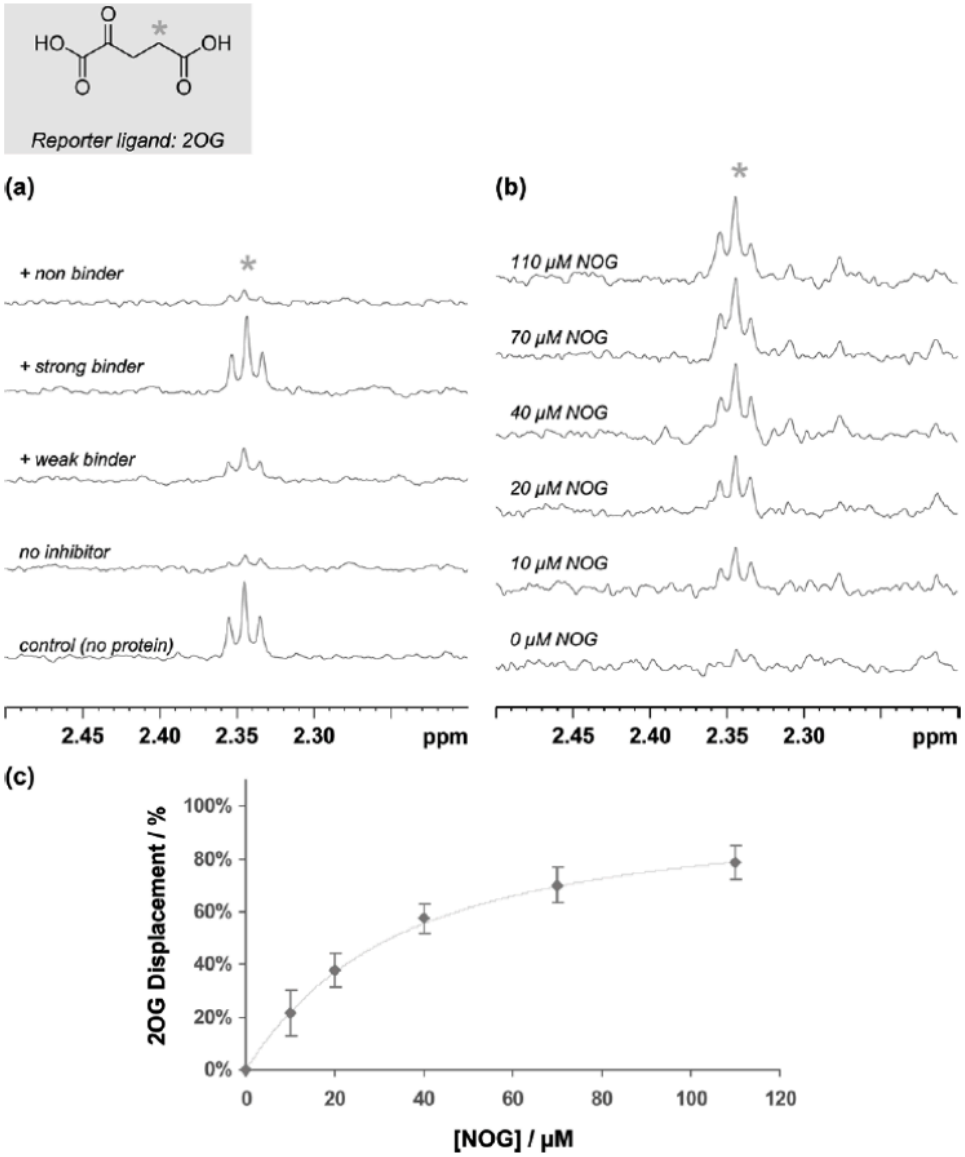

Fig. 3. Reporter ligand screening method. (a) Screening of PHD2 binders by monitoring the recovery of the reporter (2OG) signal. (b) Titration experiment monitoring reporter signal recovery at different concentrations of the ligand-of-interest (NOG). (c) Corresponding plot of the titration data. Reprinted with permission from *J. Med. Chem*. **2013**, *56*, 547–555. Copyright 2013 American Chemical Society.

$$\text{STD factor} = \frac{I_0 - I_{sat}}{I_0}$$

$$\text{STD amplification factor} = \frac{I_0 - I_{sat}}{I_0} \text{ x Ligand excess}$$

In which I_0 is the intensity of one signal in the off-resonance spectrum, I_{sat} is the intensity of the corresponding signal in the on-resonance spectrum, and $I_0 - I_{sat}$ represents the intensity of the STD spectrum.

As STD-NMR relies on fast ligand exchange between the free and protein-bound states, it cannot detect high-affinity protein-ligand systems due to slow ligand k_{off}. STD-NMR may not also detect ligands with very low affinity because the residence time of the ligand inside the protein binding site is too short for the transfer of magnetisation from the protein to the bound ligand.

The binding constant may be obtained by STD-NMR by observing changes in the STD amplification factor at different ligand concentrations.[43] However, its accuracy may be complicated by ligand rebinding, ligand relaxation time and protein saturation time. Firstly, even at modest saturation time (*e.g.* 2 seconds), the size of the observed STD signals is (partially) ordered by the ligand longitudinal relaxation time constant (T_1).[44] Secondly, ligand molecules may bind and then dissociate from the protein multiple times during the saturation time period.[43] These factors combine and contribute to an overestimation of K_D values.[43] Whilst in theory these influences may be minimised by using short protein saturation time, practically there will be problem with signal-to-noise ratio. Also, there may not be sufficient time for magnetisation to spread throughout the protein by spin-diffusion with short saturation time. In order to solve this problem, Angulo *et al.* proposed the construction binding curves using the initial growth rates of the STD amplification factors.[43] Although the protocol allows the accurate determination of K_D values, it is extremely time consuming as multiple STD spectra have to be recorded at different saturation times for each ligand concentration, and therefore the

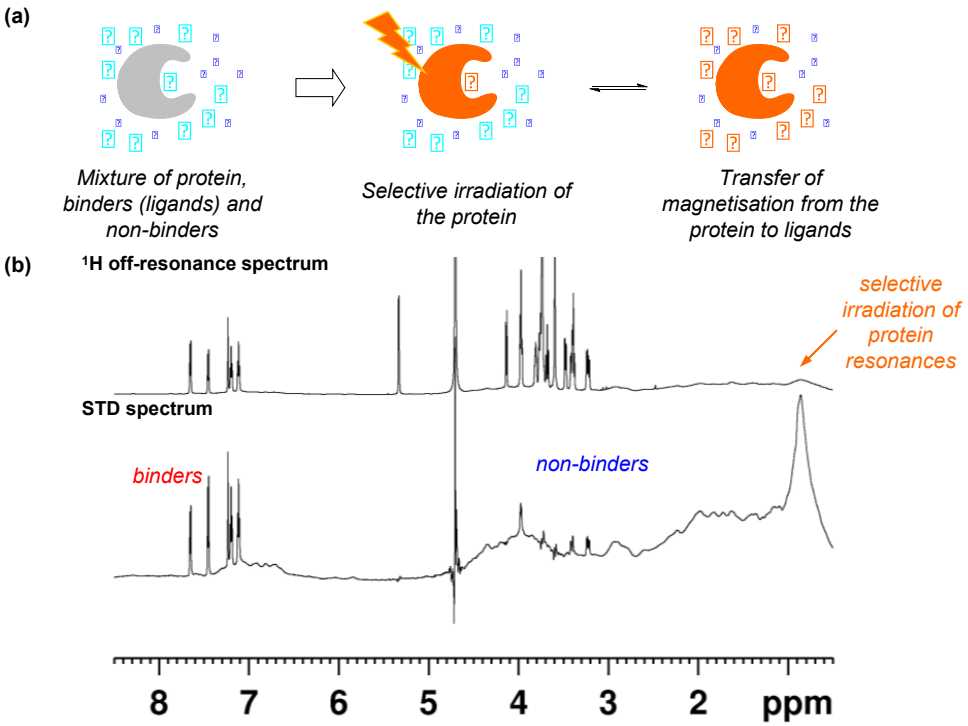

Fig. 4. (a) Scheme illustrating the principle of STD-NMR. (b) A typical STD-NMR spectrum. The mixture contained 100 μM bovine serum albumin (BSA), 10 mM tryptophan (binder) and 10 mM sucrose (non-binder). The saturation time was 2 seconds.

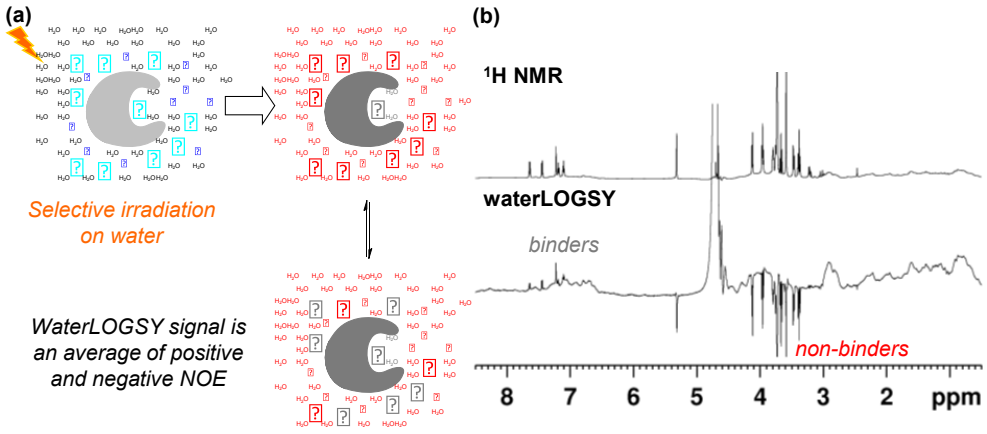

Fig. 5. (a) Scheme illustrating the principle of waterLOGSY. Red indicates positive NOE whilst grey indicates negative NOE. (b) A typical waterLOGSY spectrum. The mixture contained 100 μM bovine serum albumin (BSA), 2 mM tryptophan (binder) and 2 mM sucrose (non-binder) in 90% H_2O and 10% D_2O. The mixing time was 1 second.

use of STD-NMR to measure K_D is not widely applied.

Perhaps the most famous application of STD-NMR is the mapping of ligand binding epitope inside the protein binding site. Epitope mapping is achieved by the fact that the size of the observed STD signals generally correlate with the distance between the protein and ligand inside the binding site. However, as previously discussed, even at modest saturation time, the epitope map will be ordered by ligand T_1s rather than protein-ligand distances. Krishna and Jayalakshmi proposed the use of a complete relaxation and conformational exchange matrix analysis (CORCEMA-ST) to tackle this problem,[45] however, the method is time-consuming and require additional information such as the structural model of the protein for the full analysis. In order to simplify the analyses, Kemper *et al.* proposed the measurement of STD-NMR at saturation conditions (saturation time ~15 seconds), and then divided the observed STD enhancement of each resonance by the free ligand T_1 value of the same resonance.[46] This method thus allows one to define an accurate ligand binding epitope map from STD data without any requirement for knowledge of the protein structure.

WaterLOGSY

The waterLOGSY method is based on NOE between bulk water molecules, ligands and proteins (Fig. 5).[42] The bulk water is selectively saturated. Magnetisation then spreads in an intermolecular manner to free ligands, bound ligands and proteins, with subsequent magnetisation transfer from the protein to bound ligands. Due to differences in rotational correlation times between free and bound components of the mixture (water molecules, ligands and proteins) and hence opposite signs of their intermolecular NOEs, binders and non-binders can usually be distinguished as opposite phased resonances in the waterLOGSY spectrum.

As the bulk water is used for the transfer of magnetisation, experiments are usually conducted in 90% H_2O and

10% D_2O mixture. Typically, a NOE mixing time of 0.8 to 1 second is used for waterLOGSY experiments. A ligand excess of around 50 fold is used. This is because if the ligand excess is too large, the resulting waterLOGSY spectrum will be dominated by free ligand NOEs, whilst if the ligand excess is too small, the bound ligand concentration will be too low. Similar to STD-NMR, waterLOGSY generally does not work for very strong or very weak ligand systems, as it relies on fast exchange between free and bound ligands.

WaterLOGSY is widely used as a primary screening tool.[47] It has been shown to be a much more sensitive method than STD-NMR. The measurement of the binding constant by waterLOGSY has also been proposed, although Fielding *et al.* have shown that the accuracy may be influenced by protein concentration, although the exact reason is currently not known.[48]

Protein NMR

^{1}H-^{15}N heteronuclear single quantum correlation

The most common method to study protein-ligand interactions by protein-observed NMR is by chemical shift perturbation of backbone amide protons.[49] Analyses are usually conducted using ^{1}H-^{15}N heteronuclear single quantum correlation (HSQC) experiments. Each resonance on the HSQC spectrum represents a single amino acid (except for proline which has no NH). Side chain NH and NH_2 groups may also be present in the spectrum. Amide chemical shifts are highly sensitive to perturbations in the environment. The mapping of ligand binding sites on the protein can be readily obtained by comparing amide chemical shifts with and without the ligand. Such information is particularly useful, for instance, in establishing structural activity relationships (SAR-by-NMR) across a series of binders and ligands.[50] Provided the exchange between the ligand free and protein-bound forms is fast on the NMR time scale, it is also possible to measure K_D by following amide chemical shift changes at different ligand concentrations.[24,25]

^{1}H-^{15}N HSQC experiments usually require uniformly ^{15}N-labelled proteins, although other selective labelling schemes are also available.[51–53] For large proteins such as those with molecular weight >25 kDa,[54] ^{2}H,^{15}N-double labelled proteins may be required in order to slow down the transverse relaxation rates of the amide protons.[55] It has also been shown that at relatively high protein concentration (~1 mM), ligand binding can be monitored with unlabelled protein at natural abundance using the band-selective optimised flip-angle short-transient HMQC (SOFAST-HMQC) experiment.[56] Because amide

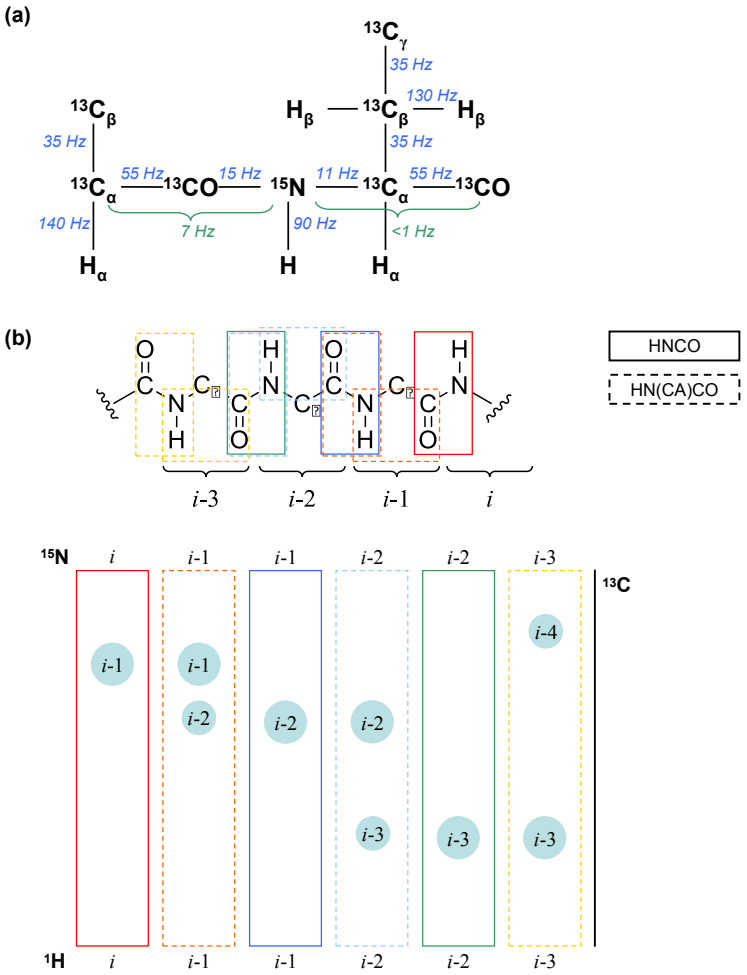

Fig. 6. (a) Typical $^1J/^2J$ coupling constants in a protein backbone spin system.[59] The blue numbers are ^1J and the green numbers are ^2J coupling constants respectively. (b) An illustration of a sequential walk along the ^{1}H-^{13}C plane of the HNCO (solid line) and NH(CA)CO (dotted line) experiments. HNCO shows correlation between the amide resonance of the current residue (*i*) and the CO resonance of the previous residue (*i*-1), whilst HN(CA)CO shows correlations between the amide resonance of the current residue (*i*) and the CO resonances of both the current (*i*) and the previous residues (*i*-1).

signals are being observed, ^{1}H-^{15}N HSQC experiments are usually conducted in 90% H_2O and 10% D_2O mixture, with a slightly acidic pH at around 6.5.

Protein backbone assignment

The bottleneck of protein NMR experiments is protein backbone assignment. For small proteins (MW < 10 kDa), the sequential assignment strategy may be used.[57] The first step involves the recording and analyses of 3D heteronuclear-edited correlation spectra (such as TOCSY-^{1}H-^{15}N HSQC) to identify amino acid spin systems by tracing through-bond scalar interactions along the amino acid side chain. The next step involves the recording and analyses of through-space dipolar interactions, usually using 3D heteronuclear-edited experiments such as the NOESY-^{1}H-^{15}N HSQC, to allow sequential NOE analysis along the protein sequence.

For larger proteins, the triple resonance assignment strategy can be used.[58,59] The minimum labelling requirement for this strategy is ^{13}C and ^{15}N. For proteins with a molecular weight of 25 kDa or above, triple labelling (with ^{2}H, ^{13}C and ^{15}N) is required.[55] Triple resonance experiments rely on the relatively large ^{1}J (and in some cases ^{2}J) couplings between ^{1}H, ^{15}N and ^{13}C in the protein backbone spin system (Fig. 6A). The first step usually involves the recording of at least one set of the triple resonance experiments, such as HN(CO)CACB, which gives correlation information between $^{1}H_i$, $^{15}N_i$, $^{13}C\alpha_{i-1}$ and $^{13}C\beta_{i-1}$, and HNCACB, which gives correlation information between $^{1}H_i$, $^{15}N_i$, $^{13}C\alpha_i$, $^{13}C\beta_i$, $^{13}C\alpha_{i-1}$ and $^{13}C\beta_{i-1}$. This allows sequential linking of the amide protons (Fig. 6B). Amino acid types can then be identified by the ^{13}C chemical shifts of α and β carbons.

Protein conformational and dynamic studies

Proteins are not rigid, and ligand binding may induce changes in the solution structure or dynamics of the protein. Protein-observed NMR can be applied to study these protein conformational changes upon ligand binding. For example, Bleijlevens *et al.* showed a conformational switch of AlkB, an enzyme that is involved in repairing damaged DNA, upon binding its cosubstrate 2OG and its coproduct succinate (Fig. 7).[60,61] ^{1}H-^{15}N HSQC shows that AlkB is unstructured in its *apo*-form, as indicated by the poor dispersion of the amide chemical shifts and resonance overlap. Upon addition of the coproduct succinate, the enzyme became slightly more folded as shown by the ^{1}H-^{15}N HSQC spectrum. Remarkably, AlkB became fully folded upon addition of the cosubstrate 2OG. This data provides a plausible mechanism for the release of succinate and replenishment of 2OG at the end of the catalytic cycle.

In addition to conformational change, protein NMR can also be applied to measure the overall and internal backbone dynamics of proteins in the nanosecond to picosecond timescale.[62,63] ^{15}N relaxation (T_1, T_2 and heteronuclear NOE) is a useful probe to characterise these dynamics because ^{15}N relaxation mainly reflects the reorientational motion of the N-H bond vector, which moves at a timescale (ns–ps) faster than the overall rotational correlation time (tens of ns). In a recent example, Ravindranathan *et al.* applied ^{15}N relaxation studies to study the influence of RNA binding to the backbone dynamics of the sterile alpha motif (SAM) domain of VTS1p, which is a posttranscriptional gene regulator in yeast.[64] The data suggests that the binding interface between the VTS1p-SAM domain and RNA became more rigid upon RNA binding. In contrast, the flexibility of the other regions on the protein domain was increased upon binding of the RNA. These experiments show that molecular dynamics could play a crucial role in modulating binding affinity and ligand recognition.

Conclusions and perspectives

In this article we have covered several NMR techniques that are commonly applied to study protein-ligand interactions, including both ligand-observed and protein-observed methods. We have described the information that these methods can provide, and also their advantages and limitations. This article is not intended to be a comprehensive review, and many emerging methods and their applications are not covered. In fact, the main focus of this article is to introduce to researchers who work at the interface between chemistry and biology, such as synthetic chemists and medicinal chemists, a flavour of

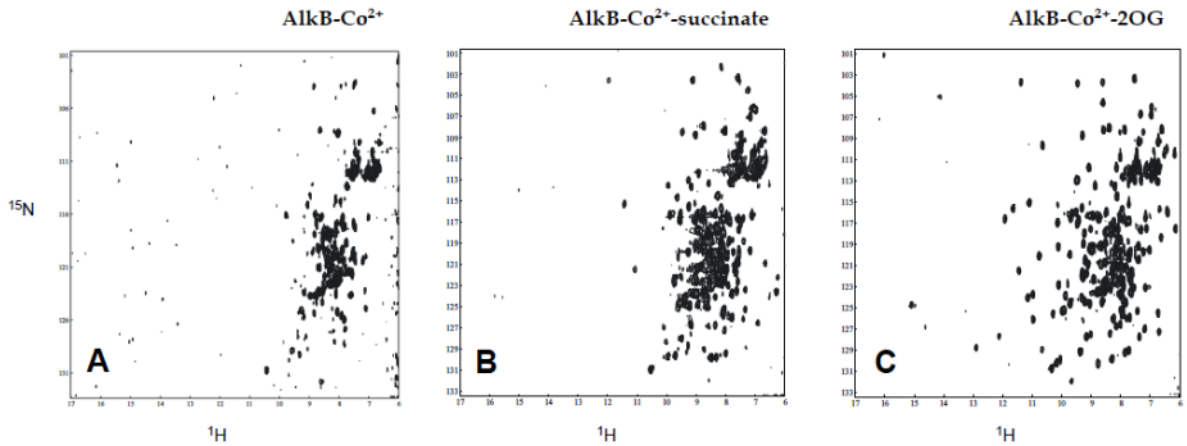

Fig. 7. ^{1}H-^{15}N HSQC spectra of AlkB. The catalytic Fe(II) is substituted by Co(II) to stop cosubstrate turnover. (a) AlkB is unstructured in the absence of its cosubstrate (2OG) or coproduct (succinate). (b) AlkB is partially structured in the presence of its coproduct succinate. (c) AlkB is fully structured in the presence of its cosubstrate 2OG. Reprinted with permission from *EMBO Rep*. **2008**, *9*, 872–877. Copyright 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

what NMR can provide in the areas of inhibitor discovery and protein-ligand interactions. Given the prevalence of NMR applications in chemical biology presented in other reviews and conferences, it is conceivable that NMR will become an even more essential tool for studying proteins, enzymes, and their interactions with ligands and inhibitors in the future.

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