NMR studies of ligand binding

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Abstract

NMR spectroscopy is an established tool in drug discovery, but its strength is commonly regarded to be largely confined to the early stages of hit discovery and fragment based drug design, where NMR offers unique capabilities of characterising the binding modes of ligand molecules that bind sufficiently weakly to be in rapid exchange between bound and free state. Here we (i) provide a meta-review of recent reviews on NMR studies of ligand binding and (ii) review recent progress towards NMR characterization of the ligand binding mode in stable protein-ligand complexes, with particular emphasis on the GPS approach enabled by paramagnetic lanthanide tags.

Introduction

NMR spectroscopy plays a prominent role in the drug discovery process in the pharmaceutical industries, first as the most important spectroscopic tool for the analysis of synthetic compounds and second for its capability to identify small ligands that bind to a specific protein target. To improve the design of a small ligand in order to arrive at a drug lead, however, the pharmaceutical chemist needs information about the exact ligand binding mode, i.e. all the interactions (hydrophobic, hydrogen bonds etc.) the ligand makes with the protein. Crystal structures and computational models deliver this information with high resolution, including water-mediated contacts. Therefore, pharmaceutical companies tend to have larger crystallography and modelling teams than NMR groups. Nonetheless, protein-ligand co-crystals usually grow very slowly and, more often than not, cannot be produced at all [1*], while modelling approaches are not very reliable, especially if the structure of the target protein is assumed to be rigid [2*]. In this situation, NMR spectroscopic methods play an important role in providing experimental information about ligand binding modes, i.e. where a ligand binds to the protein target, which conformation it assumes when it binds, in which orientation it binds and also which specific interactions it makes with the protein. This review highlights recent progress in this area for stable protein-ligand complexes.

NMR spectroscopic methods for the investigation of protein-ligand interactions have advanced significantly over the past 25 years [3,4*,5,6**], and computer power has increased even faster. Combined with computerized data analysis, even sparse NMR data can present decisive experimental information to select between different protein-ligand models generated by computer simulations. In combination, these advances give NMR spectroscopy a central role in contemporary drug discovery and design. This is reflected in the number of reviews on the topic. The authors counted no less than seven reviews published in the last 18 months [6**,7*,8*,9*,10*,11**,12*]! The following section provides a brief meta-review of these most recent reviews.

Recent reviews on protein-ligand interactions by NMR spectroscopy

The review by Oxenoid and Chou focuses on NMR of membrane proteins and how small molecule ligands modulate their conformational states [7*]. The conformations of ion channels, transporters

and G-protein-coupled receptors (GPCR) are sensitive to the binding of small compounds, which can act as agonists or antagonists.

The review by Ma et al. focuses on fragment-based drug design (FBDD) with an emphasis on the discovery of possible lead compounds binding with micromolar affinity [8*].

The review by Norton et al. focuses on ^{19}F-NMR in FBDD while pointing out the importance of using two orthogonal methods to avoid false positives [9*].

The review by Furukawa et al. points out that smaller (micromolar and even sub-micromolar) K_D values can be determined by R_2 relaxation dispersion and ZZ-exchange measurements than by conventional NMR methods that rely on faster exchange rates between free and bound ligand molecules [10*]. The authors stress the importance of knowing the exchange regime (fast, intermediate, slow) and that correct kinetic data can only be obtained by choosing the correct kinetic models, such as two- or three-state exchange models, which can be difficult and requires more than single measurements.

The review by Erlanson et al. summarizes much experience gathered in industrial laboratory settings, describing the role of NMR in ligand screening and weighing the advantages of FBDD against high-throughput screening (HTS) campaigns [11*].

The review by Gossert and Jahnke stands out as an authoritative review on current NMR techniques in drug discovery [6**]. It is particularly valuable for NMR spectroscopists as it provides optimized parameter sets for NMR pulse sequences such as STD, waterLOGSY, $T_{1\rho}$ experiments, HSQC and ^{19}F-T_2 measurements, and discusses specific pitfalls associated with different sample preparations and experiments.

The review by Teilum et al. is directed at non-NMR specialists, offering an easy-to-follow introduction into the solution NMR techniques used for measuring dissociation constants of protein-ligand complexes [12*]. For the detailed NMR theory underpinning the measurements of protein-ligand interactions, the reader is directed to the classical review by Fielding [13].

All reviews highlight the fact that NMR spectroscopy is particularly powerful for the study of kinetically labile protein-ligand complexes, so that the binding mode of the bound state can be probed indirectly by observing the NMR resonances of the free ligand. The present review focuses on NMR methods that can be applied when the exchange between bound and free ligand is too slow to allow magnetisation transfer between bound and free ligand.

Determining ligand binding modes in protein-ligand complexes

As a rule, drug discovery campaigns use unlabelled ligands because the synthesis of modified ligands is time consuming and expensive. In contrast, the target protein can often readily be labelled with isotopes or tags, especially if it can be expressed in *E. coli*. A number of well-established and recently developed NMR schemes aim for elucidating ligand binding modes in this situation.

Structure restraints from chemical shifts

Tightly binding ligand molecules change the chemical shifts of protein resonances and the binding site on the protein usually displays the largest chemical shift changes. Chemical shift changes are easy to observe but not necessarily easy to interpret. If the ligand molecule contains an aromatic ring (as most drug candidates do), it has been shown that the ring currents associated with the aromatic ring of the ligand can be used to position the ligand molecule on the protein with surprising accuracy [14]. The conversion of ligand-induced chemical shift changes into structural restraints has been refined further [15,16], but is ultimately limited by slight changes in protein structure and mobility upon ligand binding [17]. Semi-quantitative chemical shift changes also figure prominently in the analysis of protein-ligand complexes by solid-state NMR [18,19].

Structure restraints from nuclear Overhauser effects

Automatic 3D structure determination of protein-ligand complexes by NMR

Nuclear Overhauser effects (NOE) can be used to determine 3D structures of small proteins by NMR [20]. Obviously, the approach can be extended to protein-ligand complexes and the advent of increasingly powerful computers has allowed its automation. In a recent implementation, the 3D structure determination of protein-ligand complexes from NOE data has been fully automated, *including* the assignment of the protein resonances, based on double-[^{15}N,^{13}C]-filtered 2D [^{1}H,^{1}H]-NMR spectra for resonance assignment and structure calculation of the ligand, a series of [^{15}N,^{13}C]-filtered NOESY experiments recorded with different mixing times for the selective detection of ligand-protein NOEs, and the selection of an appropriate protein structure from the protein data bank (PDB) as the basis for structure computations [21**]. While the approach is very appealing, the required NMR experiments are limited in sensitivity and thus not applicable for large proteins (i.e. with a molecular weight much larger than about 15 kDa).

3D structure determination of protein-ligand complexes using sparse NOEs

Rather than attempting to define the structure of the protein-ligand complex completely from NOE data, it is more common to start from the 3D structure of the ligand-free target protein (usually determined by X-ray crystallography) and generate the structures of a range of possible protein-ligand complexes by computation, e.g. using software for ligand docking. At this stage, NMR data can add important experimental information to identify and confirm valid models. In principle, any type of NMR data can be used for this purpose. An interesting approach is to include sparse NOEs. For example, intermolecular protein-ligand NOEs can be interpreted, if the protein is made of perdeuterated amino acids with only few types of amino acids left at natural isotopic abundance. This allows interpreting the intermolecular NOEs in site-specific ways also without available sequence-specific resonance assignments of the protein (see, e.g., [22]).

Structure restraints from paramagnetism

Besides the classical restraints presented by chemical shift changes and NOEs, more recent work has focused on deriving structure restraints from paramagnetic effects, in particular pseudocontact shifts (PCS). As paramagnetic effects are still less commonly used for protein-ligand studies, this review discusses them in some detail.

Paramagnetism arises from unpaired electrons in metal ions and a few non-metal compounds such as nitroxides. Paramagnetic sites in proteins tend to affect the NMR spectrum of the entire protein, as the magnetic moment of unpaired electrons is large. The two main effects are paramagnetic relaxation enhancements (PRE), which lead to line broadening in the NMR spectra, and pseudocontact shifts (PCS), which lead to changes in chemical shifts.

Using PREs to determine ligand coordinates

All paramagnetic centres generate PREs. PREs broaden the NMR signals in a distance-dependent manner. As line broadening is easily detected by reduced NMR peak heights, qualitative PREs, usually produced by nitroxide tags, have become a popular tool to probe protein-ligand interactions [23]. To extract quantitative distance restraints from PREs, however, requires at least four spectra with good signal-to-noise ratio to measure the relaxation rate, two spectra measured of the paramagnetically labelled molecule and two of the diamagnetic reference [24]. Therefore,

quantitative measurements of PREs take longer than measurements of PCSs. Moreover, intramolecular PREs can be difficult to distinguish from non-specific intermolecular PREs. In contrast, intermolecular PCSs quickly average to zero as the nuclear spins get exposed to positive and negative values of the PCS isosurfaces, when the paramagnetic compound tumbles relative to the protein-ligand complex. This is explained in more detail below.

GPS approach to determining the binding mode of a ligand

This section discusses the use of PCSs for determining the binding mode of a ligand molecule on a protein with the help of highly paramagnetic lanthanide ions. Originally developed for ligand molecules binding to metallo-proteins with fast exchange between bound and free state [25], the concept has recently been extended to stable protein-ligand complexes [26**]. PCSs are attractive because they are long-range (40 Å and longer), straightforward to measure (namely as simple changes in chemical shift) and easy to interpret in terms of the coordinates of nuclear spins relative to the paramagnetic centre producing the PCSs. This opens an approach to structure determination, which is related to the global positioning system (GPS) used in mobile phones. Just as locating an object by GPS requires the measurement of its distance to (at least) three different satellites, the location of a nuclear spin in a protein can be determined by PCSs measured for the nuclear spin in different samples, where a paramagnetic metal ion is attached at different sites.

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Molecular GPS approach enabled by PCSs

The observation of PCSs requires the presence of a paramagnetic metal ion that is site-specifically attached to the protein. For proteins without a natural binding site for a paramagnetic metal ions, the metal ion can be provided by a complex that is site-specifically attached to the protein. In the past decade, many efforts have been directed at developing suitable metal tags, in particular lanthanide binding tags, which produce the largest PCSs. Available tags and tagging strategies have been reviewed recently [27].

A PCS is the difference in chemical shift of a nuclear spin measured in the presence and absence of a paramagnetic metal ion. PCSs are thus measured in ppm. They are largest in the vicinity of the metal ion, decreasing with $1/r^3$ with increasing distance r from the metal ion. Positive and negative PCSs can be observed, depending on the location of the nuclear spin relative

to the magnetic susceptibility anisotropy tensor ($\Delta\chi$) associated with the metal ion. The $\Delta\chi$ tensor can be viewed as an object with three orthogonal axes (χ_{xx}, χ_{yy}, χ_{zz}), i.e. the tagging of the protein with a paramagnetic metal can be regarded as equivalent to attaching a coordinate frame to the protein, with the origin of the coordinate frame located at the position of the metal ion (Figure 1). The PCS, δ^{PCS}, of a nuclear spin depends on its polar coordinates r, θ and φ with respect to this coordinate frame (equation 1).

$$\delta^{PCS} = 1/(12\pi r^3)[\Delta\chi_{ax}(3\cos^2\theta - 1) + 1.5\,\Delta\chi_{rh}\sin^2\theta\cos 2\varphi] \quad (1)$$

where $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$ are the axial and rhombic components describing the $\Delta\chi$ tensor. Equation 1 specifies the possible locations of nuclear spins with a certain PCS value, which can be depicted by a so-called isosurface. As PCSs can be positive or negative, there are positive and negative isosurfaces (Figure 1).

In analogy to conventional GPS data, the position of a nuclear spin in a protein can be determined by measuring its PCS values relative to $\Delta\chi$ tensors from metal ions attached at different sites. The location of the nuclear spin is determined by the point, where the isosurfaces associated with the respective PCSs intersect. This requires, however, that the $\Delta\chi$ tensors of the different metal sites are known, including their origin, magnitude and orientation with respect to the protein.

The parameters of a $\Delta\chi$ tensor can readily be fitted using the PCSs of at least eight nuclear spins in the protein and requires no more than the 3D coordinates of the protein and the resonance assignments of the NMR signals used for PCS measurement.

Figure 1. Structural information from PCS data. a) Superimposition of [^{15}N,^{1}H]-HSQC spectra of the Zika virus NS2B-NS3 protease modified by a lanthanide binding tag. The tag was loaded with either Tm^{3+} (blue), Tb^{3+} (red) or diamagnetic Y^{+} (black). Lines connect cross-peaks of selected backbone amides in the paramagnetic and diamagnetic samples. PCSs (measured in ppm) are very similar in the δ_1 and δ_2 dimensions, facilitating the assignment of the paramagnetic NMR spectra [27]. b) Coordinate system spanned by a $\Delta\chi$ tensor and centred about the paramagnetic metal ion. The PCS of the ^{1}H spin is described by equation 1. c) PCS isosurfaces representing the $\Delta\chi$ tensor obtained with a paramagnetic Tm^{3+} tag attached to the Zika virus protease. The isosurfaces correspond to PCSs of 1 ppm (dark blue), 0.1 ppm (light blue), −1 ppm (dark red) and −0.1 ppm (light red). The metal position is shown as a red sphere and the C^{α} atom of the tag attachment site is indicated by a green sphere. Modified from [28], copyright 2017, with permission from Elsevier.

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The first example of the GPS approach aimed to determine the binding mode of a weakly binding fragment in fast chemical exchange with the protein FKBP12. Paramagnetic lanthanide tags were attached to double cysteine motifs at three different protein sites. Although only 1-2% of the ligand were in the bound state and the average PCSs were small, the position of the ligand on the protein could be determined from the PCSs [29**]. More recently, the approach was

demonstrated for the stable complex of the dengue virus NS2B-NS3 protease (DENpro) with a covalently bound ligand [26**,30]. Three different single-cysteine mutants A–C were produced for site-specific attachment of a lanthanide tag via a disulfide bond. The $\Delta\chi$ tensors were determined from PCSs measured for backbone amide protons in ^{15}N-HSQC spectra of protein samples tagged with Tm^{3+}, Tb^{3+} and Y^{3+} (the latter provides a diamagnetic reference). With the $\Delta\chi$ tensors at hand, PCSs were measured for the bound ligand and interpreted in terms of its coordinates relative to the protein. To account for inaccuracies in measurements and metal coordinates, the coordinates of ligand moieties derived from the PCSs were indicated by localization spaces rather than single points given by the intersection between the PCS isosurfaces associated with the metal tags at sites A–C (Figure 2).

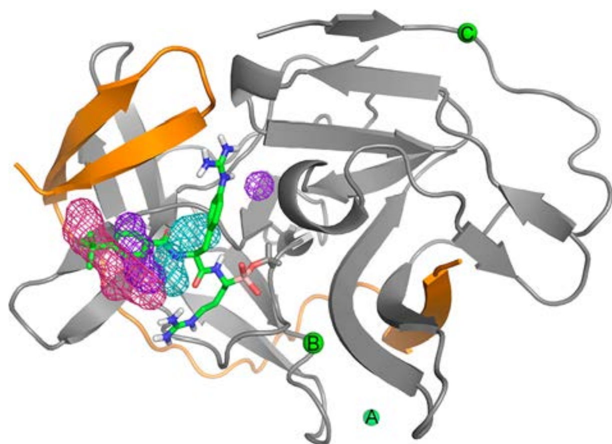

Figure 2. GPS approach to study the 27 kDa complex between DENpro and a covalently binding ligand. Green balls indicate the positions of the lanthanide ions for tags at the three different sites A–C. Meshes identify the localization spaces of the *t*-butyl group (magenta) and protons of the aromatic ring (purple and cyan) of the ligand molecule as identified by PCSs. Reprinted with permission from [26**]. Copyright (2016) American Chemical Society.

The advantage of using PCSs lies in the extraordinary quality of the correlations that can be achieved between experimental and theoretical values (calculated using Eq. 1). This translates into accurate structural information. Even PCSs measured with proteins tagged at single sites yield valuable structure restraints, albeit with less comprehensive localization information than the GPS

approach. The same applies to ligands synthesized with a lanthanide chelators, e.g. [31], where it would be difficult to position the lanthanide at very different sites relative to the ligand.

Structure restraints from combinations of NMR data

The program HADDOCK has been designed to use a wide range of unique and ambiguous NMR data as restraints for protein-ligand docking. The most recent version also accepts PCSs in the input [32*].

Selective detection of ligand signals

Once the binding mode of a ligand on its target has been established, a competition experiment with a new ligand readily yields information about whether the new ligand binds at the same site or not [33*]. In the case of fast exchange between bound and free ligand, the INPHARMA experiment can be used to determine the binding mode of the new ligand relative to that of the established ligand [34,35*]. In the case of a covalently binding ligand, however, determination of the ligand binding mode requires the assignment of NMR signals of the ligand in a sea of protein NMR signals, which is difficult unless the ligand is labelled in some way. If a ligand contains one or more fluorine atoms, as over 20% of drug molecules do, ^{19}F-NMR offers unique opportunities for its selective detection [9*,36]. Furthermore, the ^{19}F chemical shift is a sensitive reporter of specific atomic interaction groups in the protein [37]. More recently and, perhaps, more generally applicable, a *t*-butyl group has been shown to afford detection by ^{1}H NMR even in high-molecular weight systems [38*]. *t*-Butyl groups are abundant in chemical protection groups such as the Boc group. The *t*-butyl group offers the added advantage of facilitating the detection of nearby protons by NOEs with the intense ^{1}H singlet of the *t*-butyl group. In this way, the localization spaces of Figure 2 were obtained without isotope labelling of the ligand [26**].

Conclusion

Synthetic chemical tags underpin recent progress in determining ligand binding modes in stable protein-ligand complexes, by harnessing paramagnetic effects and selective detection of site-specific NMR signals of the ligand and the protein target. Importantly, the chemical tags unlock structural information independent of stable isotope labelling, which can be prohibitively expensive for many proteins and ligands. In the field of drug discovery, these tools extend the

utility of NMR spectroscopy from a role in early hit discovery, e.g. by FBDD, to the elaboration of stably binding lead compounds.

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*   of special interest

**   of outstanding interest

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