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**Interface between Microbiology and Structural
Biology as viewed by NMR**

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Abstract

NMR spectroscopy is one of two principal experimental techniques used in structural biology. It can be used to determine structures at atomic resolution and to investigate the dynamics of macromolecules and intermolecular interactions. We aim to give an overview of the use of modern high resolution NMR methodology in microbiology.

1 Introduction

NMR and X-ray crystallography are the two most powerful tools available for structure determination and have been extensively used to obtain structural information on biological molecules. The limitations of X-ray crystallography is the necessity to obtain crystals. NMR is limited by the size of the molecule and the low sensitivity of the method. Thus, these two techniques are complementary. NMR is particularly powerful for determining the structure of small molecules, for obtaining information on the dynamics of macromolecules over a wide time scale range and for studying molecular interactions. Thus, it is not surprising that it is widely used in microbiology. Indeed, NMR can be combined with isotope labelling for non-invasive analytical and quantitative analysis of metabolism, in a range of microorganisms. It has long been a critical tool for the identification of compounds and for measuring concentrations and fluxes both *in vitro* and *in vivo*.

Firstly, we will give a brief summary for scientists with no prior understanding of this method and then a few applications involving only soluble molecules will be described.

2 NMR Methodology

2-1 What is NMR spectroscopy?

Nuclear Magnetic Resonance spectroscopy quantitatively detects spin reorientation for nuclei that possess a magnetic moment in an applied magnetic field by measuring the amount of energy absorbed from a radio-frequency coil surrounding the sample. A radiofrequency field is applied to the sample and a time dependent signal can be detected and measured from the nuclear spins. This time dependent signal is then Fourier transformed to give a frequency dependent amplitude signal, corresponding to the NMR spectrum.

2-2 What are the parameters?

NMR spectra can be defined by five parameters:

- (i) chemical shifts (δ) which indicate the precise resonance frequency of the nuclei. These depend on the chemical environment of the nuclei and are expressed in a dimensionless unit, parts per million (ppm), which is independent of the strength of the magnetic field. Their ranges vary with the group and the type of nuclei. Thus, in biological molecules, ^1H chemical shifts are spread over 14 ppm, ^{13}C shifts are spread over 200 ppm and ^{15}N shifts are spread over 40 ppm.
- (ii) The spin-spin coupling which arises from weak interactions between magnetic nuclei via the electron of the chemical bond. The size of this interaction is defined by the spin-spin coupling constant (J) and is expressed in Hertz.
- (iii) The area of a signal, which is usually proportional to the number of nuclei contributing to the signal.

(iv) The longitudinal or spin-lattice relaxation time, T_1 , which is the time taken for the magnetisation longitudinal component to return equilibrium value after a perturbation.

(v) The transverse or spin-spin relaxation time, T_2 , which is the time taken for the transverse magnetisation to decay. The transverse relaxation rate ($1/T_2$) is related to the linewidth of the signal for molecules freely tumbling in solution.

There are several different mechanisms for relaxation. However, for spin $1/2$ nuclei such as proton, the major relaxation mechanism arises from dipolar interactions with other protons or nuclei. For the dipolar relaxation mechanism the relaxation rates, $1/T_1$ and $1/T_2$, depend on the τ_c the overall correlation time of the molecule and on molecular motions. τ_c characterises random molecular motions within a sample and is a function of molecular weight, of solution viscosity and of temperature. When the size of the molecule increases, both the correlation time for reorientation and the T_2 relaxation time decrease and the linewidth increases. The nuclear Overhauser effect (NOE) or dipolar cross-relaxation is related to spin relaxation. It is observed for nuclei located at a distance shorter than 5 \AA and is proportional to the inverse six power of the distance. A fixed and known reference distance allows one to associate a distance with each observed NOE. Both the spin-spin relaxation and the nuclear Overhauser effect are crucial for structure determination.

Moreover, in NMR the fundamental time scale is defined by the precession frequency of the nuclei, whereas chemical exchange phenomena allow us to define additional time scales. If a chemical group exists in different environments, the NMR parameters of the corresponding nuclei will vary according to the exchange rate. Two extremely different situations can be encountered: the fast exchange regime in which the observed parameters from all the different environments are averaged, and the slow exchange regime in which sets of parameters are observed for each environment. Finally, motions are reflected in all NMR

parameters, chemical shifts, coupling constants and relaxation rates, allowing the qualitative detection of flexibility and the quantitative analysis of relaxation data in terms of internal motions. NMR allows us to study a wide range of time scales, monitoring motions for picoseconds, seconds minutes or hours. This dynamical aspect will not be treated here, as it deserves a review on its own.

2-3 What molecule size?

Most NMR parameters are sensitive to molecular conformation. Quantification of these parameters allows structural analysis. However, this requires the resolution and the assignment of NMR signals to specific nuclei. This can be achieved easily for molecules of less than 10 000 Da by two-dimensional NMR, but is more challenging for bigger molecules. Assignment strategies based on the use of spin-spin interactions between ^{13}C and ^{15}N nuclei in isotopically enriched molecules have enabled molecules of up to about 30,000 Da to be studied, because spin-spin interactions are increased ten-fold.

Recent experiments have considerably extended the applications of NMR for structure determination. Instead of being based on distance information, they are based on orientational information. Indeed, when molecules are aligned in the magnetic field, anisotropic magnetic interactions which cannot normally be observed in high-resolution NMR spectra, can be detected (34). The two parameters measured are residual dipolar couplings and chemical shift anisotropy (CSA). The interference between dipole-dipole and CSA interactions can also be used constructively, like in TROSY experiment (33,35) which allows molecules of up to several hundred kDa to be studied. Finally, measurement of these new parameters in partially oriented media has provided new tools to increase the size of the molecules that can be studied and also the accuracy with which these structures can be determined.

2-4 Labelling

Signals from ^1H , ^{13}C , ^{15}N and ^{31}P nuclei are observable in the spectra of biological molecules. Spin-spin interactions between protons vary from few Hertz to 16 Hz when established between two or three bonds. One bond spin-spin interactions between ^{15}N or ^{13}C and their attached protons are 95 Hz and 150 Hz, respectively. When the size of the molecule increases, the linewidth increases and spin-spin interactions between protons are no longer detectable. Thus, large biomolecules can be studied by NMR by extensive isotopic labelling. For medium size molecules between 8 and 12 kDa, ^{15}N labelling is sufficient, whereas higher molecular weight molecules required double labelling, usually ^{15}N and ^{13}C . For molecules of over 30 kDa, additional labelling with deuterium further decreases the linewidth and improves the NMR spectra. Deuteriation is either selective or uniform and is often random fractional deuteriation (13). Usually, samples that have been uniformly labelled with ^{15}N and $^{15}\text{N}/^{13}\text{C}$ are prepared by growing the bacteria in minimum medium enriched with vitamins and with ^{15}N ammonium chloride and $^{13}\text{C}_6$ glucose or glycerol as the only nitrogen and carbon sources. The expression has to be sufficient, that is at least 10 mg per litre as the labelled sources are expensive. Molecules may also be selectively labelled by growing strains auxotroph for given residues in unlabelled medium supplemented with the labelled residue. For chemically synthesised small proteins of less than 80 amino acids, specific positions or amino acid types can be labelled. For other molecules several methods have been developed: RNA is generally obtained by *in vitro* transcription using T7 RNA polymerases (28) and ^{13}C and/or ^{15}N labelled ribonucleoside triphosphate is prepared from the RNA of organisms grown with ^{15}N and ^{13}C enriched nutrients (29). Methods for the large scale preparation of uniformly isotope-labelled DNA have also been developed (25).

After the molecule is labelled, its quality can be checked rapidly in 10 minutes or less by ^{15}N or ^{13}C proton heteronuclear single quantum coherence experiment (HSQC). This usually fixes the experimental conditions (temperature, pH and ionic strength). Well dispersed ^{15}N and ^1H resonances indicate a folded protein domain with segments involved in stable secondary and tertiary structures (figure 1).

2-5 Sample preparation

The sample has to be very pure and stable over time and at different temperatures because NMR spectroscopy experiments can last for several days. Proteases or nucleases would obviously destroy the sample. Impurities generally give signals sharper than macromolecules, as they are often small molecules.

NMR has intrinsically poor sensitivity and signals have to be averaged many times during an experiment because the signal-to-noise ratio increases as the square root of the number of times the signal is sampled. A few tens of milligrams of compound are necessary for structural studies depending on its molecular weight. Solutions must have a concentration of at least 1 mM and a volume of 400 μl to 600 μl is required. If Shigemi tubes (tubes with filled bottom) are used this volume decreases to between 360 μl and 250 μl . Recently, different types of small sample volume probes have also become available (i) commercial microprobes, which use small-diameter vertical sample tubes in conventional geometry probes, with volumes ranging from 120 μl to 150 μl (ii) commercial nanoprobes which produce high-resolution spectra with sample volumes of about 40 μl or less (22) (iii) and finally home-made probes which contain nanoliter to microliter-volume capillaries and consist of a solenoidal coil of about 1 mm (30). However, small sample volume probes (1 to 100 μl) are still being developed. Parallel to this technology, the cryoprobe technology has also

emerged improving the signal-to-noise ratio by a factor of 3 to 4 leading, thus reducing the amount of sample necessary for analysis and/or data collection times.

2-6 Assignments and structure determination.

Resonance assignment strategies differ significantly for each type of biomolecule, however, the strategies used are very similar and rely on the identification of connectivities via spin-spin interactions and dipolar interactions (44).

Usually, for moderately sized non-labelled molecules, two-dimensional experiments allow all signal to be assigned. For labelled molecules, assignments are obtained by combining three-dimensional ^{15}N -edited experiments and triple resonance experiments (^1H , ^{15}N , ^{13}C). These experiments are very sensitive due to the efficient transfer of magnetisation from one bond and/or two bond coupling (8).

Secondary structural elements are identified by analysing several NMR parameters: (i) induced chemical shift (CSI) (41), (ii) coupling constant values (iii) strong dipolar interactions typical of each type of secondary structures, and (iv) Apparent exchange rates of exchangeable protons with deuterium give an indication of hydrogen bond formation and of reduced accessibility to solvent.

The most important parameter used in structural determination is the nuclear Overhauser effect (NOE). All the distances derived from the quantification of NOEs are used as constraints to calculate the structure. As the precision of the calculated structure is directly related to the number of experimental restraints, it is necessary to assign several hundred NOE cross-peaks unambiguously.

3 Structural studies

The aim of this review is not to describe NMR extensively but to use a few examples to show the applications for NMR in microbiology. We chose to omit *in situ* experiments and metabolism pathways together with metabolic fluxes, as this has already been extensively reviewed recently (3). We place particular emphasis on the interface between structural biology and microbiology, where the interplay between the two communities is not always optimal.

3-1 Peptides and proteins

NMR is very effective for the determination of the structure of small proteins and many studies have been published in this field. However, NMR can be used in its full potential for the determination of the structure of the peptide domain. Indeed, peptides of less than 30 residues are often very flexible, and the rapid equilibrium between several conformers in aqueous solution precludes their crystallisation. In structural promoting environments, the number of conformations sampled can be reduced allowing the structure-function relationship to be established. In the field of microbiology high resolution NMR has been used for studies of antimicrobial peptides and of secretion signals peptides.

3-1-1 Antimicrobial peptides

Compounds obtained from natural sources, such as micro-organisms and plants, are a large number of drugs in current use, either directly or after synthetic modifications. The availability of bacterial genomic information and the increasing resistance of pathogenic organisms to conventional antibiotics means that the antimicrobial drugs need to be developed to control infection. A lot of organisms, such as animals and plants, produce offensive and defensive substances. In the invertebrates this efficient defence system often compensates for the lack of circulating immunoglobulins in vertebrates. Antimicrobial peptides are among the

substances invertebrates produce to protect themselves against invading pathogenic microorganisms. These peptides usually lyse cell membranes and each has a broad but different spectrum of antimicrobial activity (12,20). However, despite extensive studies on their mode of action, much knowledge is still needed to fully understand the basis of their selectivity. To improve potency, and specificity of antimicrobial peptides and to design cheaper and more efficient analogues we need to understand their structure-function relationships.

One family of antimicrobial peptides is the cecropin family, which consists of 35 to 37 amino acid peptides(19). Cecropins are most active against gram-negative bacteria, but they also have activity against some gram-positive bacteria. The three dimensional structure of several of them have been solved by NMR (38,18,5). They usually adopt a helix-bend-helix conformation with a highly basic amphipathic N-terminal helix and a hydrophobic C-terminal helix. The importance of amphiphilic helices has been established in a number of antibacterial and lytic peptides, and their helicoidal geometry is usually considered to be the key factor for the formation of transmembrane pores leading to cell lysis.

Cecropins are thought to be selective for prokaryotes due to the lipid composition of their membrane (14). In Shiva-3, in particular, the *in vitro* lytic effect on protozoa tested resulted in decreased growth and multiplication of *Plasmodium falciparum* and *Trypanosoma Cruzi*. These observations suggested the use of Shiva-like peptide genes to engineer malaria resistant vectors as an alternative treatment for the control of malaria (36). To improve the efficiency of this molecule its structure was studied in several media and particularly in media that mimic the membrane environment. It is composed of two helices, one amphiphilic N-terminal helix of about 20 residues long and a short hydrophobic C-terminal helix. In the presence of micelles N-terminal helix of the Shiva-3 moves much more slowly than the C-terminal helix. In particular the movement the side chains of its basic residues is restricted

suggesting that the N-terminal helix is in contact with micelles whereas the C-terminal is not (5).

3-1-2 Signal peptides

Most bacterial virulence factors that interact with host cells need to be secreted across the bacterial membrane. Thus, the various secretion systems found in gram-negative bacteria can be used as target for new therapeutics. To understand the secretion pathways we must first understand how the secretion signal function and therefore we must understand their structure.

In gram-negative bacteria, whose envelope is made of two distinct membranes, most extracellular proteins are secreted by the signal peptide-dependent general secretory pathway (GSP) which involves the N-terminal secretion signal. A other group of extracellular proteins without cleavable N-terminal signal peptide is secreted by a distinct secretion pathway involving a C-terminal secretion signal (40).

Although studies on isolated signal sequences are controversial, they have been mainly justified by the fact that signals can be functionally transferred from one protein to an other, emphasising their ability to act independently. For the N-terminal signal sequences that have been studied, conformational differences have been observed. These range from rather unordered or β -sheet structures in aqueous solution to α -helices in non polar solvents, micellar solution and phospholipid vesicles. This conformational adaptability is thought to be linked to the function of these signals (15).

Consensus structural information has been gathered on N-terminal signal sequences, however little structural information is available for C-terminal secretion signals (24,42,43). Like the N-terminal sequences their primary sequence is not highly conserved. Structural information on the C-terminal peptides of *Erwinia Chrisantemi* protease G and *Serratia*

marcescens hem acquisition protein (HasA), were obtained by use of circular dichroism and NMR spectroscopies. C-terminal signal peptides adopt an unstructured and flexible conformation in aqueous buffer solution, that switch to a helical conformation followed by a short fragment unstructured upon addition of trifluoroethanol, detergents and lipids. These studies show that the C-terminal secretion signals are composed of one α -helix followed by an unstructured C-terminal region that terminates with an essential secretion motif (42,43). This motif is composed of one negatively charged residue followed by three to five hydrophobic residues.

Structural informations could be obtained for the C-terminal peptide of HasA from *Serratia marcescens* when in its proteinic context. The last 15 residues following the C-terminal of the HasA protein are highly flexible and unstructured (21). When fragments experience motions much faster than the overall correlation time for reorientation of the macromolecule these can be detected by either homonuclear 2D experiments such as TOCSY or by measuring heteronuclear ^{15}N ^1H dipolar interactions. The high flexibility and the lack of well ordered structure of the HasA C-terminal extremity might be a prerequisite for the recognition of the protein by its ABC transporter (21).

3-1-3 Identification of small compounds

NMR is often the method of choice for identifying small unknown compounds. Microcin C7 was a “text book case” of structure determination. Many strains of the Enterobacteriaceae family produce substances with low molecular-weights or secondary metabolites with antibiotic activity (2). Microcin C7 (MccC7) is a stationary-phase product of *E. coli* that inhibits protein synthesis both *in vivo* and *in vitro* (23). MccC7 is

secreted from cells by specific dedicated machinery (30). It is a heptapeptide whose N- and C-terminal residues have been modified. The chemical structure of microcin C7 was obtained from complete assignments of ^1H , ^{13}C , ^{15}N and ^{31}P at natural abundance using homonuclear and heteronuclear two-dimensional spectroscopy. It is composed of one heptapeptide chain modified at the N-terminus by a formyl group and the C-terminus substituent consists of a phosphodiester of 5'-adenylic acid and n-aminopropanol (AMPap) linked via a phosphorus atom to an amide group, forming thus a phosphoramidate. The substituted phosphoramidate group was identified by pH titration of the phosphorous atom by means of ^{31}P chemical shift variation (17).

3-2 Oligosaccharides

Bacterial cell walls ensure cell integrity, resist mechanical constraints and have key roles in the regulation of exchanges between the cell and its surrounding environment and in the host immune response. Mycobacterial pathogenicity is often linked to the nature of the glycolipids, a constituent of bacteria cell walls. In gram negative bacteria the cell wall contains ubiquitous glycolipid molecule, the lipopolysaccharide (LPS), which is the major factor distinguishing gram-negative from gram-positive bacteria. LPS is often involved in disease and is a major surface antigen *via* its O-specific polysaccharide. Consequently, LPS specific antibodies are potential tools for the diagnosis and therapy of some of the bacterial infections such as tuberculosis, shigellosis and leprosy and it is, therefore, important to determine the structure of immunoreactive epitopes.

Although NMR data on the structure of high molecular weight oligosaccharides is more difficult to handle than structure determination of proteins of comparable weights, NMR has been particularly useful and successful in the identification of O-specific antigenic

polysaccharide chains. The main reason for this is that oligosaccharide protons have a much smaller chemical shift range (3 to 5 ppm) than proteins (about 12 ppm). The use of higher magnetic fields while increasing the signal to noise decreases the spectral overlap and can improve structural studies of these molecules. In studies on primary structure it is necessary to identify (i) the number of sugar residues in the repeating unit, (ii) the monomeric constituents and their anomeric configuration, (iii) the sequential arrangement of monomers as well as glycosidic linkage, and (iv) the presence or absence of non-glycosidic substituents. Finally, it is necessary to establish the tertiary structure and its dynamics. Usually a combination of 2D homonuclear, 1D selective excitation and 2D heteronuclear methods, allow the complete determination of quite complex polysaccharides (7).

Recently studies used NMR and molecular modelling to determine the conformational properties of a synthetic polysaccharide of *Shigella dysenteriae* type I, composed of rhamnopyranosyl, galactopyranosyl, acetamido-2deoxy α D glucopyranosyl. The conformation resembles a short, irregular spiral, with the methyl substituents on the exterior. The fact that rhamnose and galactose residues are major components of the antigenic determinant suggested that the oligosaccharide binds to the antibody via hydrophobic interactions involving three methyl groups (11).

4 Molecular interactions

NMR is a sensitive method for detecting the binding of small molecules to macromolecules, and has proven particularly valuable for the rapid identification of ligand binding sites. Indeed, the chemical shift perturbation observed for the amide proton by means of ^{15}N - ^1H heteronuclear single- and multiple-quantum correlations (HSQC or HMQC respectively) rapidly indicates where the ligand binds. A rapid and sensitive method, the

saturation transfer difference (STD) or “bioaffinity NMR spectroscopy”, has been used to identify sites at which ligands bind to macromolecules with dissociation constants of between 10^{-3} and 10^{-8} M. This method was successfully used to identify the sugar residues of the Lewis-hexasaccharide that are in contact with the fucose-binding lectin AAA protein (27). Although the method is quite powerful and easy to implement it does yield information on the receptor site of the macromolecule or on the conformation of the bound ligand. This information can only be obtained by transferred NOE techniques if the ligand binds relatively weakly ($K_D > 10^{-7}$ M) and providing that several conditions are met (9,10). Although small molecules can adopt a large variety of conformations in rapid exchange, one of these conformations is usually preferred in the bound state. Moreover, when a small molecule binds to a large molecule, even transiently, the numerical sign and magnitude of the NOEs change. Thus, it is possible to obtain an “image” of the bound ligand conformation providing great care is taken to get rid of all possible artefacts especially indirect NOE due to spin-diffusion (6, 39). This approach was successfully used to monitor the conformation of a disaccharide bound to a Fab’ fragment (1).

For tightly bound ligands ‘editing’ experiments are required to identify specifically NOEs between a ^{13}C and/or ^{15}N labelled protein and unlabelled ligand (16). If sufficient intermolecular NOEs are collected it will then be possible to dock the ligand to the macromolecule.

5 Enzyme mechanisms

NMR provides both structural and kinetic information and thus can contribute to mechanistic studies, such as substrate equilibrium, stereochemical aspect of enzyme action by

use of isotopically labelled substrates, proton transfer and stereochemical aspects of phosphoryl transfer reactions.

Perhaps one of the most successful application was the study of ionisable groups. Indeed, although X-Ray crystallography does not provide direct information on the charged state of ionisable groups NMR is the only method that can give pK values. Chemical shifts are very sensitive to the charged state of ionisable groups providing a wealth of information on protein structure in solution and on enzyme mechanisms when these residues are involved in the active site. Histidine was the first ionisable group to be studied in detail in proteins and is still the most widely studied (26).

It is possible to use NMR to obtain information on the predominant tautomeric state of histidine residues in ^{15}N labelled proteins because the chemical shifts of the ^{15}N resonances are strongly correlated with the protonation state of the imidazole nitrogen atom. Protonated nitrogen resonates approximately 170 ppm whereas deprotonated nitrogen resonates at about 250 ppm. The ^1H - ^{15}N heteronuclear multibond correlation experiment (HMBC) connected nitrogen with non-exchangeable protons from the histidine imidazole ring (figure 2). Furthermore, HMBC can also differentiate between the two neutral tautomers because the cross-peak intensities will depend on the coupling constant values (32, 4).

Concluding remarks

Although X-Ray cristallography was used to elucidate 81% of the structures in the data bank and NMR only elucidated 15 % of entries (June 2000) some of the unique features of NMR make it a valuable tool for obtaining structural information on molecules that do not crystallise due to their high flexibility and also for specific experiments that connect structural data with biochemical function and mechanism. Furthermore, recent technological advances

in the development of rapid mixing systems with highly sensitive “flow” probes for small sample volumes together with automated sample transport open the way for on–flow analysis that should aid mechanistic studies similar to those carried out on protein folding. Furthermore, the use of Shigemi microcells with cryogenic NMR probes enables the study of kinetic processes and data acquisition for transient species together with characterisation of metabolites (37). NMR spectroscopy can now follow the structural and dynamic changes taking place during intermolecular interactions and mechanistic studies in real time.

Figure 1: TROSY-HSQC ^{15}N - ^1H experiment on a 189 amino acid protein. The spectrum was recorded over 4 min with 1 scan with a 7000 Hz spectral width and 1K data point in the F_2 dimension and 64 T_1 increments with 1800 Hz in the F_1 dimension.

Figure 2: Histidine titration. A) HMBC experiment carried out on a 189 amino acid protein containing six histidines. At pH 5.6 five histidines were positively charged form whereas one is in the neutral tautomeric form suggesting a lower pK value. B) chemical shift variation of the H proton and N nitrogen for one of these histidines.

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