CHAPTER 3

Applications of the NOE in Molecular Biology

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Abstract
The nuclear Overhauser effect (NOE) arises from dipole–dipole relaxation between two spin-$1/2$ nuclei. It is therefore dependent on the distance between the nuclei and their motions. The NOE is used widely in molecular biology to measure distances, and thus to calculate structures; and also as one of a set of measurements to assess intermolecular motion. In this review, we concentrate on more recent developments of the NOE: particularly on
progress towards automation of NOE collection and calculation of macromolecular structures; on applications to study complexes and ligand binding; and on applications to hydration of macromolecules.

Key Words: NOE, Protein, Relaxation, Structure calculation, Ligand binding, Solvation.

1. INTRODUCTION

The NOE is a widely used tool within molecular biology, and it is therefore not feasible or helpful to cover every application. The two main areas in which the NOE is applied are for the generation of distance restraints in structure calculation, and as one of several measurements used to study macromolecular dynamics. Undoubtedly, the main role of the NOE is in providing distance restraints. Despite a great deal of work on other sources of structural restraints in recent years, in particular residual dipolar couplings (RDCs) and chemical shifts (which at one point almost promised to supplant NOEs as the principal source of structural information in proteins\textsuperscript{1}), NOEs remain the predominant source of structural information. We start from the assumption that all readers will have at least some familiarity with the NOE and its main applications, and we therefore begin by covering very briefly the more important aspects of NOE theory, emphasising those that will be important later. We then move on to cover each of the main areas of recent interest in turn.

In writing the review, we have had to consider where to draw the boundary of what is covered. The NOE is a phenomenon of dipolar relaxation, arising from the time dependence of dipolar coupling. We, however, felt that detailed discussion of dipolar coupling, either as applied to solids or as manifested in RDCs in liquids, was not warranted. Reviews on these topics can be found elsewhere, not least within this series.\textsuperscript{2}

There have of course been several reviews\textsuperscript{3–7} as well as two books\textsuperscript{8,9} on the NOE. For more details particularly on the theory, readers are referred to the more recent of the two books,\textsuperscript{9} or to a shorter review.\textsuperscript{10} This review mainly covers developments since the publication of the book in 2000.

2. THE BASICS

2.1. NOE theory

Nuclei \textit{in vacuo} relax extremely slowly. There are many possible sources of relaxation for molecules in a liquid or solid, but the most important of these is dipolar relaxation; that is, the magnetic field of a neighbouring nucleus acts as a source of relaxation. For many purposes, it helps to think of nearby moving nuclei producing a moving magnetic field, which effectively acts as a little local \textit{rf} pulse, rotating the nuclear magnetisation and thereby causing it macroscopically to change (i.e., the summation of many little local pulses on individual magnetisation vectors averages out to a tendency for the macroscopic vector to move in a
particular way, often to relax towards equilibrium). This effect is commonly described as *cross-relaxation*, because it arises from one spin relaxing another: spin $I$ relaxes spin $S$, and conversely, spin $S$ also relaxes spin $I$.

It is, therefore, not surprising that this relaxation is distance-dependent: the rate of relaxation that produces the NOE is proportional to $r^{-6}$, where $r$ is the internuclear distance. In more detail, for a situation in which $I$ is initially at equilibrium and $S$ is not, then the cross-relaxation rate is given by

$$\frac{dI}{dt} = -\sigma_{IS}(S - S^0)$$

and

$$\sigma_{IS} = \frac{1}{10} K^2 \tau_c \left[ \frac{6}{1 + (\omega_I + \omega_S)^2 \tau_c^2} - \frac{1}{1 + (\omega_I - \omega_S)^2 \tau_c^2} \right]$$

where $K = (\mu_0/4\pi)\gamma_I\gamma_S/r_{IS}^3$, and $(\mu_0/4\pi)$ is a proportionality constant to get everything into the correct units, $h$ is Planck’s constant divided by $2\pi$, $\gamma_I$ and $\gamma_S$ are the gyromagnetic ratios for nuclei $I$ and $S$, $r_{IS}$ is the internuclear distance, $\omega_I$ and $\omega_S$ are the Larmor precession frequencies of nuclei $I$ and $S$, and $\tau_c$ is the correlation time of the $IS$ vector, meaning the characteristic time for reorientation of the vector and thus approximately the time taken for it to move by one radian. Thus the relaxation rate depends on the type of nuclei involved, on their distance, and on the correlation time for the $IS$ vector. Because of the $\omega_I$ and $\omega_S$ terms, it also depends on the frequency of the NMR signals, and thus on the field of the spectrometer used. We will consider each of these effects.

The simplest case to consider is one where there is only a single correlation time throughout the molecule, or in other words where the molecule is rigid, and its motion is isotropic, that is, it tumbles at the same rate in all directions, which implies that it must be close to spherical. If we consider $^1$H-$^1$H relaxation for proteins in a 600 MHz spectrometer (for which $\tau_c$ is of the order of 10 ns), then $\omega_I$ and $\omega_S$ are very similar and are both close to $6 \times 10^8 \times 2\pi$ or $4 \times 10^9$, implying that the first term in the bracket in Equation (2) is negligible compared to the second. This means that the cross-relaxation rate is negative (a ‘negative NOE’). There are a number of consequences of this, but the most striking is that the magnetisation vectors of $I$ and $S$ relax together but in opposite directions, that is, the intensities of $I$ and $S$ converge. This is an intuitively easy situation to understand, and means that cross-relaxation leads to magnetisation being spread around the molecule essentially in a diffusive process. This process is known as *spin diffusion* and will be discussed in more detail below.

For all protons in the rigid protein, we can therefore simplify the above equation to

$$\sigma_{IS} = cr_{IS}^{-6}$$

Somewhat confusingly, a negative cross-relaxation rate (a negative NOE, as found in proteins) corresponds to a peak in a 2D NOESY spectrum of the same sign as the diagonal (a positive peak as normally drawn) whereas a positive cross-relaxation rate (a positive NOE, as found in small molecules and also in ROESY) corresponds to a NOESY peak of opposite sign to the diagonal.
Thus, the cross-relaxation rate is very simply proportional to \( r^{-6} \). The cross-relaxation rate is normally measured as the intensity of a NOESY cross peak at short mixing time (the time delay during the NOESY sequence during which NOEs build up), from which we conclude that the intensity of a NOESY cross peak is proportional to \( r^{-6} \). This then allows us to measure distances within the molecule, as long as we have some reference calibration distance with a measured NOE intensity \( A_{\text{ref}} \):

\[
r_{IS} = r_{\text{ref}} \left[ \frac{A_{IS}}{A_{\text{ref}}} \right]^{-1/6}
\]

This is the basis on which all distance measurements are made using the NOE. The best reference distance to use is one that roughly matches the distance to be measured. In particular, use of a very short reference distance, such as that between two methylene protons in a \( \text{CH}_2 \) group, leads to errors and should be avoided.

Of course, if the protein is not rigid or not isotropic, then this equation is no longer valid. In fact, the isotropic requirement is usually not a problem, since most proteins rotate isotropically to within a factor of 2, and the \( r^{-1/6} \) dependence means that this only introduces an error into the distance measurement of \( 2^{-1/6} \) or less, which is only 1.1. However, the rigidity is more problematic. Local internal motion means that different parts of a protein can have very different correlation times, which can introduce reasonably large errors into the distance calibration. Internal motion generally results in short local correlation times in mobile regions, which makes the cross-relaxation rate slower: in other words, NOEs become small or even disappear. This may result in a lack of restraints, but as long as the restraints are binned in the conventional way into strong/medium/weak (Section 3.2) then this merely results in a weaker restraint than necessary.

Nonetheless, these problems are as nothing compared to the problems introduced by spin diffusion, which form the biggest difficulty in relating peak intensity to distance. Spin diffusion means that in a chain of spins, the NOE spreads along the chain, in a manner which can be thought of conveniently as the spread of heat from one container to another. It weakens as it goes, but the effect is that in the presence of spin diffusion, NOE intensities no longer reflect distance accurately: distant spins appear closer than they really are, and conversely to a lesser extent close spins appear farther than they really are. This is the reason why in almost all structure calculations made using restraints derived from NOE cross-peak intensities, the distance restraints do not correspond to the single distance predicted from Equation (4). This effect can be very severe, as illustrated in Figure 1, and there is no simple way to know whether a given NOE is affected by spin diffusion or not. In principle, one can measure NOEs using a series of mixing times, but this approach is time-consuming, especially as accurate intensities need to be obtained particularly for the least sensitive short mixing times, and in practice this is seldom done. The magnitude of the spin diffusion effect can be calculated exactly, so if one knows the geometry of the spins, then one can calculate exactly how the NOE will behave. However, it is usually the converse situation that applies: we have the measured NOEs and we want to use these to
determine the molecular structure. A number of approaches have been used to overcome the problem, some of which are presented below. However, for almost all practical applications, no attempt is made to correct for spin diffusion other than by weakening the distance restraints.

There is a large scaling effect that arises from the correlation time. The correlation time scales approximately as the molecular weight, meaning that small molecules have very short correlation times, of around \(10^{-11}\) s or less, whereas proteins have correlation times of 5 ns or longer. The effect on the cross-relaxation rate is shown in Figure 2. The main conclusion to be drawn from the figure is that as the correlation time (molecular weight) increases, cross-relaxation gets faster, and therefore NOEs build up faster, in principle permitting NOESY experiments of even very large molecules provided the NOESY mixing time is short enough. The figure has an unusual appearance because it plots the absolute value of the log of \(\sigma_{IS}\) against the log of \(\tau_c\). The absolute value is used because at small \(\omega \tau_c\) the cross-relaxation rate is positive (i.e., the first term of Equation (2) is larger than the second), while at large \(\omega \tau_c\) it is negative, as we have seen already. At a value of

![Image of Figure 1](image-url)

**Figure 1** The effect of spin diffusion on NOE-derived distances. Protons \(I\) and \(S\) are 4.0 Å apart, and there is a third spin \(X\) equidistant between \(S\) and \(I\). Spin diffusion from \(I\) to \(S\) via \(X\) leads to NOE buildup faster than would occur in the absence of \(X\). The figure shows results for three positions of \(X\) with distances \(r_{IX} = r_{XS}\) of 2.5 Å (dotted line), 3.0 Å (dashed line), and 4.0 Å (solid line). The time course of \(IS\) NOESY cross-peak buildup is shown, calculated for a protein with a correlation time of 10 ns on a 600 MHz spectrometer. For \(r_{IX} = 4\) Å the \(IS\) NOE builds up smoothly and is hardly affected by the presence of \(X\). However, for \(r_{IX} = 2.5\) Å there is a ‘lag time’ of about 25 ms, during which \(X\) has little effect on the NOE between \(I\) and \(S\), but then the \(IS\) NOE buildup rate increases rapidly because of spin diffusion via \(X\). The NOE intensity at 100 ms was used to obtain an apparent distance \(r_{IS}\) using Equation (4): for \(r_{IX} = 2.5, 3.0\) and 4.0 Å these apparent distances are respectively 3.5, 3.9 and 4.0 Å. Spin diffusion therefore has a marked effect on the apparent distance when a third spin comes between \(I\) and \(S\). Calculated using iterative numerical integration.11
of just greater than 1, the cross-relaxation rate goes from being positive to being negative (Figure 3). In other words, for small molecules, the NOE builds up very slowly and the cross-relaxation rate is positive, while for proteins the NOE builds up rapidly and the cross-relaxation rate is negative. For molecules of medium size (corresponding to small unstructured peptides in water), the NOE crosses over from positive to negative, and thus NOEs are very small. This effect is very important for studying both ligand binding and hydration. In both cases, we have situations where the intermolecular vector (protein to ligand or protein to water) has a very rapid correlation time, and so the cross-relaxation rate is small and positive. It should therefore be easily possible to distinguish between protein–protein NOEs (large and negative) and protein–ligand NOEs. The implications of this are discussed below.

The lack of any NOE for intermediate sized molecules, as discussed earlier, can be countered by using a technique called ROE (rotating frame NOE), in which cross-relaxation occurs not in the z (longitudinal) direction but in the transverse plane. ROEs are non-zero for all correlation times (Figure 3). ROE experiments are achieved by spin-locking magnetisation using pulse trains and therefore are prone to both COSY-type and TOCSY-type artefacts, as well as intensity variation because of off-resonance effects, which lead to cross peaks far away from the

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carrier having a mixture of ROE and NOE. In addition, because for proteins $T_2$ relaxation is faster than $T_1$ relaxation, signal-to-noise in ROESY spectra tends to be less than in NOESY. Therefore ROESY is not often used for proteins (whereas it is used extensively for saccharides, for example): the most common reason for using ROESY is that spin diffusion is essentially non-existent in ROESY.

2.2. Macromolecular structure determination

Ever since the introduction of triple resonance spectra using $^{13}$C and $^{15}$N double labelled protein in the 1980s, the standard procedure for calculation of protein structures (and very largely for nucleic acids also) has been:

- Use triple resonance backbone experiments (such as HNCO, HN(CA)CO, CBCA(CO)NH, HN(CACB, HNCA) to assign the protein backbone
- Use HC(C)H and CCH TOCSY or similar experiments to assign sidechain signals
- Run NOESY spectra and peak pick to obtain a clean list of NOE cross peaks, from which noise and artefacts have been removed
- Assign the NOE peaks and use these as restraints for structure calculation

These two last steps are usually carried out iteratively: the initial peak list will contain a large number of ambiguous peaks (i.e., peaks where the assignment of one or both protons is not unique because of chemical shift degeneracy), which are set on one side initially, and very crude structures are calculated using only the unambiguous peaks. The crude structures are then used to filter the ambiguous peaks, because some of the possible ambiguous assignments are clearly not consistent with any possible structure, thereby allowing some of the previously
ambiguous peaks to be assigned. This then permits a better structure to be calculated and so on. In addition, it is invariably found that some of the confidently assigned NOEs used from the start violate consistently, which prompts detailed examination of these restraints, followed by possible reassignment; that is, the peak picking will also require some revision in the light of the structures and their restraint violations. The restraint set is then gradually refined, gradually increasing the number of NOEs used, after which a ‘final’ structure is calculated. There is no obvious end point to this process: typically it continues until the person carrying it out loses the will to live, this being a tedious process in the extreme. The structure is then validated.14 As described in an excellent review by Nilges14a (quoting an earlier review by Thornton and colleagues 15), there are four possible types of validation that could be carried out:

1. How well does the structure fit to the data used to calculate it? This could be done using an $R$-factor16 (see Ref. 17 for a good review of this topic) but is more commonly done merely by listing restraint violations
2. How well does the structure predict restraints not used in the calculation? (routinely done in crystallographic studies using the free $R$ factor but very rarely done in NMR, because of the difficulties inherent in predicting a spectrum from a structure, including problems in predicting chemical shifts and accurately calculating NOEs in the presence of spin diffusion and unknown internal motion; not to mention the fact that one cannot usually afford to set restraints aside merely for the purpose of cross-validation)
3. How well does the structure match to what we know about protein structures? (done mainly using programs such as PROCHECK18 and WHATIF19, but also using deviations of bond lengths and angles from standard distribution)
4. How well does the structure explain the biology? (done with very varying amounts of detail depending on what is known about the biology)

These steps have been refined a little in the 23 years since the first NMR structure was published20 (where of course homonuclear spectra only were used, but this makes very little difference), but have remained essentially the same. Examples can be found widely in all the major journals in biochemistry and structural biology. We, therefore, present merely one protein example, the membrane protein OmpX, chosen to illustrate some interesting features.

Outer membrane protein X (OmpX) is, as its name suggests, a transmembrane protein: it is a 148-residue E. coli protein that promotes cell adhesion and invasion of host cells, and is involved in defence against the host complement system. This makes it an important target for structural determination, because of the very low number of structures of membrane proteins, but it also makes it a difficult target. Membrane proteins are usually difficult to purify, and need solubilisation using detergent micelles, which is often problematic, both because the spectral quality of reconstituted proteins can be poor and the solubility and stability can be limited, and because the solubilised protein micelle is of high molecular weight. Before the start of the NMR study, a crystal structure was published showing OmpX to have an eight-stranded $\beta$-barrel structure.
OmpX was overexpressed in *E. coli* as inclusion bodies (insoluble aggregates). It was grown either as fully $^{15}$N, $^{13}$C, $^2$H labelled or else with selective $^1$H labelling of isoleucine, valine and leucine methyls, by growth on medium that included $\alpha$-ketobutyrate and $\alpha$-ketovalerate. The carbon source was $^{13}$C,$^2$H-glucose, employed in order to obtain as complete a level of deuteration as possible. It was resolubilised in 5 M guanidine hydrochloride, which denatures the protein, and then slowly diluted into 3% dihexanoylphosphatidylcholine (DHPC), dialysed and concentrated to about 2 mM protein. This produced micelles of approximately 60 kDa, with a correlation time of approximately 25 ns. This detergent at this concentration produced the best spectrum of several tested, using TROSY HSQC spectra. TROSY experiments were used to obtain backbone and sidechain methyl assignments. NOEs were measured from 3D $^{13}$C or $^{15}$N-filtered NOESY spectra, with mixing times of 300 ms. Such a remarkably long mixing time can be used without excessive spin diffusion because of the extensive deuteration of the protein, though one suspects there must have been spin diffusion even so, and that use of such a long mixing time was a necessity because of weak NOEs. $^{13}$C$\alpha$ and $^{13}$C$\beta$ shifts provided information on secondary structure using TALOS, while $d_{NN}$ NOEs provided the register of the $\beta$-sheets. This provided 107 distance restraints. These restraints alone were sufficient to reproduce the $\beta$-barrel reasonably well, though the loops at both ends, and particularly some long loops at the extracellular end, were essentially undefined. One would not normally expect this small number of NOEs to produce a result even as good as this, but the fact that the protein is a continuous $\beta$-barrel, and therefore its secondary structure is completely defined by hydrogen bonds across the strands, is a big bonus. One would not expect an $\alpha$-helical protein to be defined so well only by HN–HN NOEs. The global RMSD for the $\beta$-barrel was 3.1 Å (Figure 4A) but improved dramatically to 0.9 Å on addition of restraints on 100 hydrogen bonds across the $\beta$-sheets based on the limits to the sheets indicated by NOEs and carbon shifts. Again, this is rather a drastic step: one would not normally want to rely so heavily on hydrogen bonds to improve structure quality, but it was presumably a necessity in the absence of any other restraints. (Now, one would no doubt try measuring RDCs.) The resulting structure resembled closely the X-ray structure.

The sidechain methyl-labelled sample was used to acquire separate $^{13}$C and $^{15}$N-resolved NOESY spectra, this time with the more respectable but still long mixing times of 200 ms, and the additional restraints improved the structure, giving NOEs between methyl groups that were up to 7 Å apart in the structure. There were a total of 526 NOEs (still a very small number compared to what would be expected for a water-soluble protein of comparable size). The NOEs were this time supplemented by only 34 hydrogen bonds, a much more respectable number, made possible by the more complete NOE set. The resultant RMSD was 0.93 Å for the best defined residues within the $\beta$-barrel, and 1.17 Å for all residues in secondary structure. The residues in loops were much less well defined (Figure 4B). By comparison to the RMSD for the HN-HN only structure, restrained using 100 hydrogen bonds (0.9 Å) it is clear that hydrogen bonds...
achieve a drastic tightening of the structure, and therefore need to be used with caution.

In addition to the expected intramolecular NOEs, NOEs to the lipid detergent were also seen, both to the hydrocarbon tails and to the choline methyls. This elegantly defined the limits of the detergent-enclosed part of the protein, with a large number of NOEs from both surface methyls and HN protons to the hydrocarbon tails, and a small number of NOEs to the choline headgroup (Figure 4C). The observation of only a single set of resonances for all DHPC molecules additionally indicated rapid exchange of molecules in contact with the protein, but the negative cross-relaxation rate to DHPC indicated residence times longer than about 1 ns (see Section 5).

A very interesting category of protein structure determination has been the study of unfolded proteins: do they have any structure, and if so what is it? Long-range NOEs (in sequence, not distance) can be seen for unfolded proteins in solution, both for proteins unfolded by denaturant and for natively unfolded proteins. Clearly, this indicates contacts between residues far apart in the sequence, and the presence of hydrophobic clusters. The problem is that the $r^{-6}$ dependence of the NOE means that transient contacts can give rise to significant NOEs, even when the corresponding structure is not highly populated. Simulations demonstrate that NOEs are not very good indicators of the distribution of conformations present, and remarkably that ensembles of highly non-native structures can give averaged NOEs very similar to those of a folded structure. It is clear that structural studies of unfolded proteins need to consider a range of

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**Figure 4** The structure of the *E. coli* membrane protein OmpX. (A) The structure as determined by NMR using only 107 HN–HN NOEs. (B) The structure as determined using 526 NOEs to amide protons and methyl groups, plus restraints for 34 hydrogen bonds. (C) A space-filling view of OmpX. Residues that have NOEs to the detergent hydrocarbon chain are shaded in dark grey. Residues with NOEs to the detergent choline headgroup are shaded black. The dashed lines indicate the expected position of the boundary between the hydrophobic and hydrophilic surfaces, corresponding to the expected membrane boundary. The lines are 28 Å apart. Figures prepared using Pymol, using protein coordinates 1orm and 1q9f deposited in the Protein Data Bank.
experimental variables. A large fraction of expressed proteins contain sections that appear to be natively unfolded, and further development of these methods is therefore important.

Structures of saccharides are in many ways more difficult than proteins. The \( ^1H \) signals are crowded and close to the water; there are few long-range NOEs, implying that distances have to be measured extremely accurately to provide useful structural information; and correlation times are frequently variable (implying problems in applying Equation (4) directly) and in the range where NOEs are close to the zero-crossing point. This tends to mean that ROE experiments are used much more widely for saccharides than they are for proteins or nucleic acids, and often using the ‘T-ROESY’ version that removes TOCSY cross-peak intensity.

### 2.3. Dynamics from relaxation measurements

The second main application of the NOE in molecular biology is to studying internal dynamics in proteins. In most such applications, we are studying not \( ^1H-^1H \) NOEs but \((^1H-^{15}N)\) NOEs in the H–N bond. Here also, the cross-relaxation rate varies markedly with the correlation time, as shown in Figure 5. In particular, for a short correlation time, the NOE to \(^{15}N\) from \(^1H\) is large and negative, while for long correlation time it is close to zero. We still use an equation similar to Equation (2), but now the internuclear distance is fixed (because only the directly bonded proton contributes appreciably to the NOE), and what we are measuring is the effect of the correlation time on the cross-relaxation rate. In particular, the standard ‘model-free’ assumption is that the NH bond is subject to two different motions: the overall tumbling of the protein, with correlation time \( \tau_c \) and amplitude \( S^2 \) (where \( S^2 \) is a number ranging from 0 to 1), and a faster local motion, with correlation time \( \tau_e \) and amplitude \((1 - S^2)\). The value of the NOE alone is not enough to determine both \( S^2 \) and \( \tau_e \), so the \(^{15}N\) \( T_1 \) and \( T_2 \) are also measured. From a combination of these values, estimates of \( \tau_c, \tau_e \) and \( S^2 \) can be obtained.

It may be helpful here to note that the ‘model free’ assumption is model-free in that it does not require any particular model of the type of motion occurring. It does, however, implicitly make a number of assumptions which may not always be true (in particular independence of different motions and the separability of global and local motions), and the numbers derived are therefore more to be viewed as a simple numerical view of the degree of internal motion than an accurate portrayal of internal motion.

The NOE is a cross-relaxation phenomenon, and all NOEs are therefore sensitive to molecular motion. This creates some very interesting possibilities for experimental measurements. NOEs between any two nuclei can be measured, and are sensitive to motions at different frequencies, depending on the two nuclei studied. For example, \(^{13}C-^{13}C\) cross-relaxation in fully labelled proteins turns out to be relatively easy to measure and to analyse, and provides information on

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\[ \text{Equation (2)} \]

In most publications dealing with \(^{15}N\) NOEs, the long-correlation-time value is drawn as 1 rather than 0. The difference comes from whether you consider the change in intensity (giving a value of 0) or the actual intensity (giving a value of 1). This difference is of long standing and it is not worth trying to change the convention.
sidechain mobility.\(^{39}\) Even \(^1\text{H}\)–\(^1\text{H}\) NOEs can be used to provide information on mobility, in a particularly elegant experiment.\(^{40}\) As described earlier, for proteins the NOE (longitudinal) cross-relaxation rate is negative, while the ROE (transverse) cross-relaxation rate is positive. One can construct pulse sequences in which cross-relaxation occurs partly longitudinally and partly transverse, the relative proportions being determined by the spectroscopist. This is most simply done by using off-resonance ROESY, in which the effective tilt of the relaxation frame is described by an angle \(\theta\). For each internuclear vector, there must exist an angle \(\theta_0\) at which the total cross-relaxation rate (and therefore the observed NOE) is zero. This will depend only on the motion of the vector, and is most sensitive to

**Figure 5** The dependence of the NOE on \(\omega \tau_c\) for an ideal two-spin system following steady-state saturation of \(S\). The zero crossing point for homonuclear NOE is at \(\omega \tau_c = 1.12\), and the curves are calculated for a 500 MHz spectrometer (\(\omega_H = 500 \times 2\pi \times 10^6\)). The curves are shown for \(X(1\text{H})\) with \(X = 1\text{H}\) (homonuclear, solid line), \(^{13}\text{C}\) (dashed and dotted line), \(^{15}\text{N}\) (dotted line), and \(^{19}\text{F}\) (dashed line). The curves for \(^1\text{H}\) and \(^{19}\text{F}\) are very similar because of the similarity of their gyromagnetic ratios.
motions on a timescale of 100–300 ps. Different values of $\theta_0$ are observed for different parts of the protein, thereby demonstrating differential mobility on a ps timescale. The method does not require an isotopically labelled molecule, making it suitable for analysis of motion in samples that are difficult to label, such as saccharides.

3. FASTER AND BETTER

In recent years, there has been a strong emphasis on structural genomics projects, which have received large amounts of funding. The aim of these projects is to determine protein structures rapidly, and to set up automated processes for doing so, so as to increase the throughput rate as much as possible. Both X-ray and NMR communities have risen to the challenge, and there has been a lot of activity.\textsuperscript{17,41–47} Many authors have commented that the traditional manual methods for NOE assignment and structure refinement as described earlier tend to be subjective and difficult to validate, whereas automated methods are objective and reproducible – and of course if the automation is done properly, the structures should be at least as good as any manually derived structures and probably better. It is therefore of great importance that automated methods be developed and validated.\textsuperscript{45}

In order to calculate a protein structure by NMR, one has first to assign the spectrum. This is normally done using a set of backbone (and C$\beta$/H$\beta$) triple resonance experiments, usually followed by sidechain assignment based on $^{13}$C TOCSY, all of which experiments rely only on scalar couplings. All of the assignment methods are based on matching up frequencies in different triple resonance experiments and sorting them to obtain a self-consistent set of assignment. There have been many developments to speed up acquisition of these spectra, which do not fall within the remit of this review. Once the chemical shift assignment is complete, there is then conventionally a second and quite separate stage, in which $^1$H–$^1$H NOESY experiments are used to obtain distance restraints. NOESY experiments are acquired, following which there is a process of peak picking: deciding which local maximum is a real peak and which is noise or artefact (caused, for example, by being close to the diagonal or to the residual water peak). After this, the NOE peaks must be assigned. The assignment of the NOEs is based again on matching chemical shifts: for each NOESY cross peak, its chemical shifts are measured and matched up to an assignment table, with some tolerance being imposed such that the chemical shift of the NOE peak must match the tabulated value within a tolerance. Clearly, too tight a tolerance may result in the correct assignment being missed because of small changes in the chemical shift due to different sample conditions, deuterium isotope effects, sample degradation, etc., while too loose a tolerance will result in very large numbers of possible assignments. Finally, the assigned NOEs are used as distance restraints to calculate a structure. By far the slowest, most tedious and most error-prone process in this list is the NOE assignment. There are opportunities for speeding up and improving all of these steps, but particularly NOE assignment.
Ever since the first structure calculations, it has been understood that this conceptually simple linear process is not in practice viable or sensible. A large majority of NOE cross peaks cannot be assigned unambiguously, and the small number of unambiguously assigned peaks are usually not enough to define the global protein fold, let alone to calculate the structure. One must therefore somehow use the information from the ambiguous peaks, most obviously by some kind of iteration between structure calculation and NOE assignment. Different groups have come up with different solutions to this problem, as outlined below. In some approaches, it is not even necessary to carry out chemical shift assignments before starting on analysis of NOEs. We look at these aspects in turn.

3.1. Faster acquisition

Most NOESY spectra of biomolecules have been and continue to be 3D spectra. Compared to 2D spectra, 3D spectra have better resolution and are thus easier to assign and to measure peak intensities (necessary for distance quantitation). It has been estimated that only a few percent of NOEs in medium-sized proteins can be assigned directly by chemical shift matching in 2D spectra, whereas 3D spectra allow ca. 20% to be assigned immediately.\(^{48}\) We should note that already this creates some problems, since in order to obtain a 3D spectrum there must be two magnetisation transfer processes: one via the NOE, but the other via \(J\)-coupling. Thus, cross-peak intensities in a 3D spectrum are not determined entirely by the NOE but depend also on the size of \(J\)-couplings and on relaxation during the magnetisation transfer. They are also harder to measure than intensities in a 2D spectrum. This of course makes the relationship between peak intensity and distance even more ill-defined.

Acquisition of a 3D spectrum requires time incrementation in two indirect dimensions, and thus unavoidably takes longer, and/or implies worse resolution, than a 2D spectrum. Nevertheless, in 3D spectra, one can still obtain reasonably good resolution in all three dimensions. We wish to find NOEs between all pairs of protons in the protein, both those bonded to N and those bonded to C. A 3D NOESY spectrum is normally acquired as a NOESY-HSQC,\(^{49}\) in which the coherence transfer process is

\[
\text{H}_A(t_1) \xrightarrow{\text{NOE}} \text{H}_B \xrightarrow{1J} \text{X}_B(t_2) \xrightarrow{1J} \text{H}_B(t_3)
\]

where \(X\) is either \(^{13}\text{C}\) or \(^{15}\text{N}\), \(X_B\) is directly bonded to \(\text{H}_B\), and the third transfer step is required merely for observation of the signal on \(^1\text{H}\). It is reasonably straightforward to set up an experiment that acquires data on both \(^{13}\text{C}-\text{H}\) and \(^{15}\text{N}-\text{H}\) systems simultaneously, speeding up the acquisition.\(^{50}\) In a 3D spectrum, \(\text{H}_B\) is defined by both a \(^1\text{H}\) frequency and a \(^{13}\text{C}\) or \(^{15}\text{N}\) frequency, and is thus reasonably unambiguous (meaning that for an average size protein there may be 4 or 5 possibilities within the chemical shift tolerance), while \(\text{H}_A\) only has a \(^1\text{H}\) frequency, and is thus much less well defined. One may then try to find the ‘matching’ \(\text{H}_B-\text{H}_A-X_A\) peak elsewhere in the spectrum, but the process is neither simple nor unambiguous.
An obvious solution to the difficulty is to use a 4D spectrum, in which one would have the corresponding X frequency for both protons, and therefore a far less ambiguous restraint. The problem here is that to acquire a 4D spectrum requires very long acquisition, and still results in rather low resolution in several dimensions, as well as low sensitivity because of magnetisation losses due to relaxation during the transfer steps. A number of ingenious solutions have been proposed in recent years.

One is to use reduced dimensionality, in particular the so-called G-matrix Fourier transform (GFT) method. The idea here is to acquire several indirect dimensions jointly, as sums or differences of frequencies, and then work back to the original frequencies. Thus to acquire a standard 3D experiment, it is necessary to acquire, for example, 128 points in one indirect dimension ($\omega_1$) and 64 in the other ($\omega_2$), giving a total of $128 \times 64$ 1D FIDs to be acquired. By contrast, reduced dimensionality requires the acquisition of only $128 \times 7$ FIDs, a time saving of 9. This is done by acquiring seven 2D spectra with indirect frequencies of $0, \omega_1, \omega_2, \omega_1 + \omega_2, \omega_1 - \omega_2, -\omega_1 + \omega_2,$ and $-\omega_1 - \omega_2.$ These seven spectra then need to be combined together by adding and subtracting different combinations in order to obtain the contributing frequencies, $\omega_1$ and $\omega_2.$ The resultant data have better definition of contributing chemical shifts than one would get from the 3D, because each frequency has been measured multiple times. The benefits are of course not completely unmixed: there is an added complexity in the processing, and the total acquisition time is shorter, implying that the resultant sensitivity is lower. However, the point has been made that in a modern high-field spectrometer equipped with a cryoprobe, sensitivity is often excellent, and therefore one does not need long acquisition times for sensitivity reasons. The time savings are even greater for higher dimensionality (e.g., compressing five dimensions into two or three). However, for NOESY, probably the most useful application is for the compression of a 4D NOESY into three dimensions, allowing a 4D spectrum to be acquired in as little as 30 h. The complexity of the processing in GFT (and its patenting) has so far deterred spectroscopists from applying GFT extensively, but it is starting to be taken up more widely.

There are a number of related concepts, of which the most popular are Hadamard techniques and spectral reconstruction from tilted planes. In Hadamard spectroscopy, one effectively only acquires indirect frequencies where data are expected: thus rather than acquiring 128 indirect frequencies, one only measures a lesser number of frequencies, namely those where (based, for example, on a quick 2D spectrum) one expects signals. This is an efficient method for small molecules where there are a small number of indirect frequencies, but is less applicable to large molecules. Spectral reconstruction is a reworking of the projection reconstruction method used in X-ray tomography. One acquires 2D projections of 3D spectra, and given enough suitably chosen projections at different angles one can back-calculate the peak positions in the original 3D spectrum. As with GFT, one needs far fewer projections than one would to acquire the standard 3D spectrum. Wüthrich and colleagues have developed the method into a technique named APSY, which has permitted a 6D spectrum to be acquired as a series of projections in two dimensions. So far, it would be fair to say that the idea is in the category of ‘interesting idea, let’s wait and see if it works’, rather than being an immediately applicable method.
A drawback of Fourier transforms as a way of extracting frequencies from FIDs is that one requires linear sampling of the data – for example, 128 regular increments of $t_1$, all with the same number of scans. A more efficient way of acquiring data is to acquire more points or more data at early increments, where the signal-to-noise is better, for example to apply an ‘exponential weighting’ to sampling. This however means that one cannot use an FT to process the data. But there are several alternatives available, particularly three-way decomposition\textsuperscript{57,58} and maximum entropy,\textsuperscript{59} both of which are promising ways of significantly speeding up acquisition. Four-dimensional decomposition has been applied to acquisition of 4D NOESY spectra, resulting in a 6-fold reduction in acquisition time.\textsuperscript{60} Related methods involving sparse sampling have been proposed recently.\textsuperscript{61–63}

3.2. NOE peak assignment and structure calculation

Particularly in a structural genomics era, a key question is, what is the most efficient way of carrying out the iterative process of protein structure calculation and NOE peak assignments? Most calculations use a highly ambiguous set of NOEs to calculate an imprecise and probably inaccurate initial structure, and then use the initial structure to weed out some possible assignments as being so far apart in the structure as to be impossible. This produces a less ambiguous set of NOEs, which makes a better second round structure, and so on. There is general agreement that an inaccurate initial structure biases most assignment procedures, and leads to an inaccurate final structure, implying that it is important to get the initial structure right. There is universal agreement that no initial set of NOE assignments (particularly one picked automatically) will be error-free: there will always be some assignments that turn out to be wrong (i.e., not present in the correct structure), because noise has been picked as a real peak, or there are missing chemical shift assignments, or because of chemical shift variation between samples etc. Is it worth spending time to unambiguously assign as many NOE peaks as possible at the start, so as to generate better initial structures? What is the best way of using ambiguous and possibly incorrect NOEs? There are of course several answers to this question.

A popular program for structure calculation is ARIA (Ambiguous Restraints for Iterative Assignment),\textsuperscript{64} now in version 2.2. Like all the other popular software, it uses an unedited initial list of (mostly ambiguous) NOEs and gradually refines the list during the structure calculation. At the start of the calculation, it filters the list by rejecting any NOE peak that does not correspond to an assigned proton (or heteroatom) frequency within a certain user-defined chemical shift tolerance. It then uses the NOEs to calculate an initial structure. An important feature of ARIA is that ambiguous NOEs are applied using ambiguous distance restraints:\textsuperscript{65} if there are two possible assignments for an NOE cross peak, then the restraint is given by

$$d_{\text{ambig}} = (\Sigma d^{-6})^{-1/6}$$  \hspace{1cm} (5)
In other words, an ambiguous cross peak is treated as having contributions from all its possible assignments. If the ambiguous restraint is satisfied by one of the assignments within the list of possibilities, then it is satisfied, and the other possibilities have no effect. This idea has been used very widely and seems to work very well. Nilges and colleagues (the authors of ARIA) have also addressed the question posed earlier: is it worth working hard to assign as many NOEs as possible at the start of the calculation? They demonstrate that the key requirement is that the ambiguous NOE list for each cross peak should contain the correct assignment: this is far more important than limiting the number of NOEs that are ambiguously defined, as long as the iterative assignment converges slowly (in a simulated annealing method, this means that it cools slowly). In other words, it is better to use chemical shift tolerances for cross-peak assignment that are large enough not to exclude the correct assignment, and then compensate for the highly ambiguous restraints by converging slowly. They present methods to estimate how large these parameters should be. A similar conclusion was reached by other authors. ARIA has a number of other interesting features, including a final refinement step in a shell of water molecules, which improves the geometry of the structure without any major detrimental effect on NOE violations.

The standard procedure for calibration of distances from NOE intensities has been to use a range of reference distances within the structure, preferably ones of similar distance to those to be measured, and then to generate conservative (i.e., wide) upper and lower bound ranges based on the reference intensities; the reason being that spin diffusion and internal dynamics mean that it is difficult if not impossible to accurately relate intensity to distance: spin diffusion in particular means that some long distances will have reasonably intense NOEs, whereas a variety of relaxation effects mean that some short distances will have small or zero NOEs. Typically, the lower bound is set equal to the closest possible approach (van der Waals contact or approximately 2 Å), while the upper bound is set to 3, 4, 5 or 6 Å depending on whether the NOE is classified as strong, medium, weak or very weak. There is no good reason for this strong/medium/weak classification—it is merely a simple scheme, based on the approach used in the first protein structure determination by NMR. Automated methods permit a more sensible method of distance calibration. In ARIA for example, the overall calibration is based on an analysis of all NOE intensities observed together with statistical analysis of average distances expected in proteins, while the upper and lower bounds are typically set to target \(d \pm 0.125 \ d_{\text{avg}}\). In fact, the errors in NOE-derived distances are not distributed normally (as implied by this equation) and it is more correct (and produces better structures) to use a log-normal distribution, or at the very least to use a calibration error based on real distributions in proteins. More recently, the possibility has been introduced into ARIA to correct distance restraints for spin diffusion, based on intermediate structures. This approach has been used for a long time in connection with nucleic acid structures, where the low density of protons and their uneven distribution throughout the structure means that spin diffusion is a particular problem.

The other major software for structure calculation is a suite of programs based on DYANA, incorporated both into an overarching environment called
KUJIRA\textsuperscript{43} and into what is currently the only truly fully automated structure calculation program, FLYA.\textsuperscript{74} This tackles the problem in a slightly different way. In particular, it uses two methods called network-anchoring and constraint-combination (now also available in ARIA). Network-anchoring makes use of the observation that a close contact between two residues is usually manifested as a set of NOEs between different protons on the two residues not just one: it therefore weights NOEs so as to put more weight on NOEs that are supported by a network of others. Constraint-combination is an interesting and successful way of mitigating the effects of incorrect NOE assignments (arising from picked noise peaks, chemical shift variability, etc.): NOEs are selected randomly in pairs (or more) and the restraint is applied as a single ambiguous restraint. In this way, the probability that an incorrect restraint will bias the structure is greatly reduced. There follows an iterative procedure (as done also by ARIA), in which the restraints are used to calculate an initial structure, and the structure is used as a filter to reject NOE assignments that exceed the restraint distance in some defined fraction of the structure calculations by more than a defined cutoff. These slightly less ambiguous restraints are used as input for the next round, and so on. In this way, the degree of ambiguity of the NOE restraints is gradually reduced. In ARIA, the filter is made stronger on each iteration, on the grounds that the structures get better on each round.\textsuperscript{5} The consequences of these filtering procedures in both ARIA and CANDID is that a significant proportion of NOE cross peaks are never used in the final round, because the possible assignments are all inconsistent with the structure. Güntert (the author of CANDID and CYANA) has suggested that the criterion for a good structure produced by CANDID is that fewer than 25\% of long-range NOEs should have been rejected by the final round.\textsuperscript{42} Programs differ in how easy it is for the user to find out which initial NOE cross peaks were rejected.

There are several other programs in use. NOAH\textsuperscript{75,76} attacks the problem of ambiguous NOEs by ignoring any ambiguous NOEs with more than two possible assignments, randomly selecting individual assignments from the list, using them as restraints, and randomly exchanging between assignments throughout the calculation. This procedure works, essentially because correct NOE restraints work together to converge on a single structure, whereas incorrect restraints pull in different directions and therefore do not converge.\textsuperscript{42} AUTOSTRUCTURE\textsuperscript{77,78} uses an approach similar to network-anchoring in that it searches for patterns of NOEs typical of different secondary structure elements, which are more reliable than individual assignments – an idea that has been around for a long time,\textsuperscript{79} and demonstrates that good ideas never go away, they just reappear in a different form! The simulated annealing program XPLOR-NIH has been modified to include this concept, into the program PASD, where it has been shown to produce structures that are not influenced by errors in the global fold of structures generated in earlier iterations (unlike ARIA and CANDID for example).\textsuperscript{80} KNOWNOE\textsuperscript{16} uses a Bayesian approach to eliminate NOE restraints from a list of ambiguous restraints, based on a distance cutoff. This makes the first-pass structure rather accurate, compared to other approaches, but it suffers from the problem that chemical shifts in the picked list need to be very accurate. SANE\textsuperscript{81} is very similar to ARIA, except that it uses the AMBER forcefield, and deliberately requires the user to reject violated NOEs.
One of the problems confronted by all automated assignment programs is that chemical shift assignment of the spectrum is never complete. That is, there are always some nuclei for which there is no assignment, and therefore any procedure that rejects NOEs by matching to frequencies of known assignments will automatically reject the NOE, even though it may be a perfectly valid NOE, and may well be of value to chemical shift assignment. Indeed, CANDID requires ~90% complete assignment to work reliably,\(^{42}\) and AUTOSTRUCTURE requires about 85%.\(^{17}\) A recent paper shows that unassigned resonances can be represented as unattached atoms, which can subsequently be assigned and thereby attached to the protein, generating better structures and allowing structure calculations using only backbone and H\(\beta\) assignments.\(^{82}\)

Another thorny question that has long been debated is the value of including ‘non-NOEs’ as restraints: if an NOE is not observed, is it valid to include it with a lower bound of approximately 4 Å, to stop the distance becoming too short? This is a risky thing to do, because NOEs can be missing for all sorts of reasons, in particular line broadening. Nevertheless, if NOEs are observed to other protons, then there cannot be line broadening and it should be justified to use non-NOEs as restraints. So far the issue has been raised several times,\(^{5,83,84}\) but has not been seriously examined in automation procedures.

Oligomeric proteins generate particular problems with automated structure calculation, because in general one does not know whether any NOEs seen are intramolecular or intermolecular. The most general solution to the problem appears to be to use ambiguous NOE restraints in conjunction with symmetry restraints,\(^{5}\) and/or (for dimers only) to use differential labelling of the monomers.\(^{85}\) There have however been some recent developments, including a method to identify which NOEs are important for distinguishing between different inter-subunit assignments, and hence which ones need to be treated carefully.\(^{86,87}\)

### 3.3. Using NOEs without prior peak assignment

The backbone assignment step is now rapid and almost or completely automatic. However, sidechain assignment remains at best semiautomatic, and can involve considerable human intervention.\(^{17}\) Therefore, for rapid and maximally automated structure calculation, is it necessary to carry out peak assignment (particularly of sidechains) before starting on the structure calculation? Do NOE spectra (supported by other knowledge such as assignments of homologous proteins) contain enough information to both assign and calculate structures? There have been several attempts in this direction, with some hope that they may succeed.\(^{45}\)

Grishaev and Llinas\(^{88–91}\) have done a good deal of work in this area. The idea of their programs, named BACUS, ABACUS and CLOUDS, is that it should be possible to use the NOE spectra in the absence of a complete assignment to generate distance maps (‘clouds’) between protons. Of course, it helps to have more information, so backbone triple resonance spectra are very helpful.\(^{90}\) The assignment of peaks and structure calculation is done iteratively, crucially using Bayesian statistics to make the most probable assignments.\(^{89}\) The method works on spectra containing little or no peak overlap, and perhaps in consequence has
been quoted as working well in conjunction with reduced dimensionality (GFT) data.\textsuperscript{46}

A very interesting approach is to use homology information together with other easily available information, typically backbone assignments and RDCs to provide an initial and rather crude structure calculation, and use this to help assign otherwise unassigned NOE spectra. Meiler and Baker\textsuperscript{92} used their structure prediction program ROSETTA to calculate a large ensemble of possible structures. The structures were ranked for their fit to experimental NOE cross peaks and RDCs. The best structure(s) were then used as the initial structure, from which NOEs were assigned iteratively. The method was tested on nine small proteins, and gave the correct fold in all cases, although success was heavily dependent on the quality of the initial assignments. In four of the nine, it produced good structures. A related application uses ROSETTA in combination with chemical shift values, with remarkable success.\textsuperscript{93} The combination of shifts with limited NOE information is particularly promising. Clearly, as modelling and \textit{ab initio} methods for predicting structures improve, the generation of initial structures from very limited NOE sets (and other experimental information) will also improve, possibly leading to major improvements in automated structure calculations.\textsuperscript{94–97}

A Monte Carlo program has been developed for protein assignment that uses NOEs together with triple resonance $J$-coupling experiments.\textsuperscript{98} Importantly, it also uses assignments from structurally related proteins (e.g., assignments of the free protein to study complexes). Another program has been described that uses only $^{15}$N-labelled protein with RDCs and HN–HN NOEs.\textsuperscript{99,100} This is likely to become an increasingly common situation. The programs were only used for assignment, but clearly one could integrate this with the other methods described in this section for automatic structure calculation in the absence of sidechain assignments. In principle, NOEs alone could provide all the information needed for assignment,\textsuperscript{101} though one suspects that this would only work on very small proteins.

A related topic is structure calculation of very large proteins. For large proteins, it is necessary to perdeuterate the protein almost entirely, to reduce relaxation and permit the successful application of triple resonance experiments for backbone assignment.\textsuperscript{102} The only remaining protons are HN protons, necessary for sensitive observation, but HN alone do not generally provide enough density of protons for structure calculation (although crude structures can be generated this way,\textsuperscript{103} and it has been demonstrated that HN NOEs plus a good set of RDCs can produce good structures, at least for small proteins\textsuperscript{104,105}). It was therefore suggested to protonate methyl groups from isoleucine, leucine and valine, by growing the protein on $^{13}$C-labelled $\alpha$-isoketobutyrate and $\alpha$-ketovalerate.\textsuperscript{106} This strategy has proved very successful. Interestingly, proteins containing only HN and methyl protons have few relaxation routes and therefore rather little spin diffusion. This permits NOEs to be transmitted over very large distances, of up to 12 Å, but obviously requiring a longer mixing time than normal.\textsuperscript{107} It was shown that the NOEs from methyl groups and HN alone are sufficient to calculate rather crude structures, in a largely automated manner, but only with the aid of RDCs.\textsuperscript{44}
In smaller proteins, methyl–methyl NOEs can be observed with good sensitivity without perdeuteration and used to generate global folds rapidly.\textsuperscript{108}

This review largely ignores nucleic acids. This is not because nucleic acids are any less interesting or important than proteins, but merely because there has been little development on the application of NOEs to nucleic acids: by and large assignments and structure calculations carry on being done in roughly the same, low-throughput manner as they have always been. RNA creates particular problems because the structure is not predictably linear in the way that almost all DNA structures are, and therefore standard methods for assignment based on the expected sequential NOEs between the sugar protons of one nucleotide and the $H8/H6$ proton of the next are not always reliable. There have been some developments in this area.\textsuperscript{109,110}

4. COMPLEXES

Although the main application of the NOE is to calculation of the structures of biomolecules, principally proteins, the NOE has also been used extensively to study complexes: mainly those of proteins with other proteins or with low molecular weight ligands, but also complexes of nucleic acids. Tightly bound complexes behave in exactly the same way as single proteins, but weaker binding presents additional opportunities and problems. There is a recent review, dealing mainly with small molecules and including a discussion of diffusion-based methods.\textsuperscript{111}

4.1. The transferred NOE

Proteins are large molecules and therefore have short correlation times, rapid NOE buildup and extensive spin diffusion. By contrast, ligands are usually small molecules and therefore have slow NOE buildup and no spin diffusion. This implies that if a NOESY experiment is carried out on a system of protein plus ligand, where the ligand is in excess and (as is commonly the case) the off-rate is fast (implying binding in the $\mu$M range or weaker),\textsuperscript{8} then NOEs within the free ligand develop very slowly, whereas NOEs within the bound ligand develop much more rapidly. Exchange of the ligand between bound and free will then produce free ligand (with sharp signals and chemical shifts at the positions of free ligand) displaying NOEs characteristic of the bound state. This is a very useful experiment, since it provides conformational information on the bound ligand but the information is measured from the easily observed and assigned free ligand signals, and is known as the transferred NOE (trNOE)\textsuperscript{4,8} or exchange-transferred NOE.\textsuperscript{112} It has been widely used. A particular beauty of the trNOE is that because the cross-relaxation rate increases with correlation time, it actually works better for larger proteins. Larger proteins have the additional benefit that their signals tend to be very broad due to rapid $T_2$ relaxation, and therefore interfere with the ligand signals less than they do in small proteins. The trNOE has been applied to
ligand binding to systems as large as ribosomes, tRNA or the acetyl choline receptor solubilised in a micelle. More commonly, it is applied to protein/ligand complexes. In favourable cases, the ligand conformation can be defined reasonably accurately, which may allow the structure of the complex to be modelled using simple docking methods. This is presumably because structural complementarity and energy calculations can often define the structure of a protein/ligand complex rather well given even limited experimental data, an observation made in the context of STD.

Initial applications of the trNOE were (in retrospect) overinterpreted, claiming accuracies for the bound conformation that are not realistic. The pendulum then swung the other way, leading many people to avoid the trNOE. However, in recent years interest in the trNOE has reawakened, not least through the availability of programs for simulating the trNOE, and the trNOE has once again emerged as an extremely useful tool for determining the structure of bound ligands. There are several precautions that need to be taken to ensure that the data are realistic. Spin diffusion within the bound ligand is just as much of a problem as is spin diffusion within a protein, and similar methods need to be taken, such as using short mixing times in the NOE experiment. The ‘dilution’ of bound signals by the excess of free ligand does, however, permit a longer mixing time than one would use for the protein alone. Spin diffusion from bound ligand to protein and back again does affect the intensities of trNOEs, but the effect is usually not large. This is the case in particular for ‘proton-rich’ ligands such as peptides, whereas carbohydrates can be more problematic. The trROESY experiment has been proposed for this purpose (e.g., see Ref. 120), but is not generally useful because the difference in cross-relaxation rate between ligand and protein is small, and therefore the excess of free ligand has to be very small. A particularly insidious problem for the trNOE is secondary weak binding to alternative sites, and it is necessary to check for this, most obviously by the addition of strong competitive ligands that should displace the ligand and remove any observed trNOE.

In several cases, observations have been made on proteins that bind two ligands simultaneously, in which case it is often possible to observe trNOEs between one ligand and the other, thereby of course not only showing that both ligands can bind but giving structural information on their relative orientation. This is a powerful technique and should be of significant benefit to ‘SAR by NMR’ strategies, in which a tight-binding ligand is conceptually assembled by joining together two weak-binding ligands that bind at adjacent sites. More remarkably, it is even possible to observe NOEs between ligands when both ligands bind at the same site, but competitively (i.e., they cannot both bind together). In this case, magnetisation is transferred from one ligand to the other via the protein.

As described earlier, trNOE works because cross-relaxation in the bound state is much faster than cross-relaxation in the free state. The same is also true of cross-correlated relaxation, which can provide information on dihedral angles in the bound state. Joint use of both methods can in favourable cases give better bound structures, but the range of exchange rates that are suitable is rather limited.
4.2. STD and cross saturation

Saturation of a protein resonance followed by spin diffusion means that after a few seconds of saturation, the protein resonances are almost completely saturated. It does not matter much at what chemical shift value the protein resonances are irradiated, since spin diffusion will distribute saturation efficiently, at least for larger proteins. This saturation can then be transferred to a bound ligand. In a similar way as the trNOE, exchange of the ligand to the free state will then result in a ligand signal that is attenuated because of the spin diffusion. Therefore, if a difference spectrum is calculated (off-resonance protein saturation minus on-resonance), the result is that signals can be seen for any ligand that is bound to the protein and that has an off-rate sufficiently fast for the free signal to be observed (i.e., an off-rate faster than $T_1$ relaxation of the free ligand). This effect is known as saturation transfer difference or STD, and has proved very effective as a method for screening libraries of small molecules for binding to a protein target and for detecting weak binding. A very similar method has been used for assigning bound ligand signals.

Somewhat more controversial is the use of STD for epitope mapping, that is, to define which part of the ligand is in contact with the protein. The concept is very simple: the parts of the ligand that are closest to the protein will have the largest NOEs. Therefore by measuring the percentage of ligand signal lost on saturation of the protein (or more commonly the percentage of signal seen in the difference spectrum), it should be possible to work out where the ligand binds. The problem is that $T_1$ relaxation of the ligand resonance is in competition with spin diffusion: the intensity of the STD therefore depends markedly on the relaxation rate of the ligand proton. Therefore if the ligand signals all have similar relaxation rates, as in many saccharides and peptides, epitope mapping is probably reasonably secure: otherwise it would be prudent to use great care, in particular to measure $T_1$ for free ligand and use simulations to help interpret the spectra.

A related method has been used for studying protein–protein binding, and has been named cross-saturation. Saturation of any resonance in protein $A$ leads, by spin diffusion, to saturation of all signals in the protein. If $A$ binds to another protein $B$, then saturation will spread to this second protein also. This could provide a way to identify a binding partner, and characterise its binding site. However, there are two problems to overcome: how to saturate resonances on $A$ without also saturating $B$; and how to avoid spin diffusion on $B$, which would spread saturation all over $B$. Both these problems were neatly overcome by using $^{15}$N labelled and perdeuterated $B$, and unlabelled $A$, and detecting the saturation on $B$ using a difference HSQC or TROSY. This means that saturation of $A$ is specific to $A$ and that the proton density on $B$ is low enough that spin diffusion is severely restricted, thereby allowing identification of the binding site. There is a requirement for almost 100% perdeuteration of $B$, which is not easy to achieve given that growth of proteins in D$_2$O but on U-13C glucose as sole carbon source usually results in retention of a reasonably high fraction of the glucose protons as $^1$H (as little as 3% residual $^1$H is enough to compromise the experiment). In addition, spin diffusion is preferably kept small by using up to 50%...
D$_2$O as solvent, resulting in lower sensitivity. It is probably this requirement for very high levels of deuteration that has meant that the technique has not been widely adopted. The method has also been applied to protein–nucleic acid binding.\textsuperscript{138} Here it is in some respects simpler, because there are regions of the spectrum (in particular the high-field end) where it is possible to saturate the protein without risk of saturating nucleic acid also.

4.3. Labelling

The trNOE and STD require dissociation of the ligand from the protein, and are thus limited to off-rates of about 100 s$^{-1}$ or faster, and therefore effectively to dissociation constants of micromolar or weaker. To study more tightly bound complexes, one has to find some way to observe the bound ligand signal selectively, of which the most obvious is by a differential isotope labelling strategy. Such a strategy is also applicable to more weakly bound complexes and to protein–protein complexes, as we have seen already with cross-saturation.

The obvious way to do this is to label the protein with $^{15}$N and/or $^{13}$C, and observe the NOE to the ligand using an isotope filtering scheme for detection of NOEs to $^{14}$N-H and/or $^{12}$C-H. Better results are obtained by selective inversion of the water resonance in the middle of the mixing time, to suppress indirect relaxation pathways.\textsuperscript{139} This would ideally require selective excitation of protein signals, which is difficult to do because $^1J_{CH}$ varies quite widely, implying that simple isotope-selective spin echoes are not very effective. However, it is possible to achieve reasonably good inversion of protein resonances across a range of $^1J_{CH}$ using adiabatic $^{13}$C pulses.\textsuperscript{140} An elegant STD experiment has been described that uses perdeuterated but methyl-protonated protein, and works even in the absence of detailed protein assignments.\textsuperscript{141} An application of selective protein labelling has been described that characterises the ligand conformation and requires a known structure of the free protein but does not require assignment of the protein spectrum.\textsuperscript{142}

Protein–protein complexes are in many ways simpler than protein–ligand complexes, since both components are rather rigid, and the contact area is large: hence there are relatively few orientations in which the complex can be assembled. They can therefore be studied using rather few intermolecular NOEs (as little as one), preferably in combination with RDCs to define the relative orientation of the proteins;\textsuperscript{143} or by cross-saturation and RDCs.\textsuperscript{144} Alternatively, and in the spirit of some of the automated joint assignment/structure calculation methods described earlier, it is possible to model the protein–protein complex, predict intermolecular NOEs, and then iteratively assign the experimentally observed NOEs aided by the prediction.\textsuperscript{145} For protein–protein complexes, the reverse approach (detection on $^{13}$C-H only) is also possible, and of course provides one way of studying otherwise symmetrical protein dimers.\textsuperscript{146} A labelling strategy using a uniformly $^{13}$C-labelled protein in complex with a fully deuterated but methyl-protonated partner has also been described.\textsuperscript{147} Selective labelling provides a good approach to studying RNA–protein complexes, in this case using base-specific labelling.\textsuperscript{148,149}
4.4. Exchange regimes

Reference has been made to exchange rates several times already, because clearly these are vital to studies of molecular complexes. The NOE is a consequence of cross-relaxation, and in order for an NOE to be observable on a free component, cross-relaxation has to occur faster than the off-rate. This implies that trNOE techniques such as trNOE and STD are only applicable for relatively weakly bound complexes with dissociation constants weaker than low micromolar. Very often, this is the sort of dissociation constant where binding is just starting to get biologically interesting, and annoyingly trNOE and STD disappear for strongly bound ligands. Competition with even tighter binding ligands has been used to ‘recover’ trNOE and STD signals.

Exchange can affect the success of experiments in other ways too, most significantly because intermediate exchange (i.e., exchange where the rate of exchange is comparable to the difference in chemical shift between free and bound species) causes very significant line broadening. Again, this tends to occur at low μM dissociation constant, and can be a serious problem in characterising complexes by NMR. The most obvious way to solve the problem is to modify the ligand (or the protein) so that binding is weaker and exchange is faster. Another solution is to use an excess of ligand. In this case, the broadening of the ligand signal is reduced, but on the other hand the net NOE on the ligand is smaller. A solution to this is to use one-dimensional driven NOEs, in conjunction with perdeuteration/methyl protonation, necessary to minimise spin diffusion during the long saturation period.

5. INTERACTIONS WITH WATER

Bulk water, of course, has a very short correlation time because of its rapid translational diffusion, while water buried within the protein interior often has a much longer correlation time. This means that NOEs from protein to bulk water also have a short correlation time while NOEs to tightly bound waters have long correlation times, and implies that it should be possible to characterise water bound to proteins, and to measure its lifetime, within certain limits. This is important, since proteins only function in water, and most functionally important interactions occur within the thin hydration layer around the protein.

Bulk water exchanges rapidly with water molecules bound to proteins, and it also exchanges rapidly with most hydroxyl groups in proteins, meaning that all waters and almost all hydroxyls resonate at the same chemical shift. On the very reasonable assumption that proteins are surrounded by a hydration layer that has different properties than bulk water, then there are three main mechanisms by which cross peaks can be observed between protein and water in NOESY or ROESY spectra (Figure 6), consisting of different combinations of exchange and/or NOE. Chemical exchange between two protons (e.g., Figure 6D) invariably gives rise to positive peaks in both NOESY and ROESY spectra. An NOE between two protein protons, as in Figure 6C, gives a positive peak in NOESY but
a negative peak in ROESY (i.e., negative and positive cross-relaxation rates, respectively). The interesting case is an NOE between a protein proton and a water molecule, as in Figure 6A, B. If the correlation time for the interaction is long (Figure 6A), then it behaves like a protein–protein NOE and has a positive peak in NOESY and a negative peak in ROESY, whereas if the correlation time is short (Figure 6B), then it has a negative peak in both. Therefore (as summarised in Figure 6), a direct NOE between protein and water always gives negative peaks in ROESY, but gives positive peaks in NOESY if the residence time of the water is long, and negative peaks if the residence time is short. Situation (c) (an exchange-relayed NOE) has positive peaks in NOESY and negative peaks in ROESY. And situation (d) (direct exchange) has positive peaks in both. Therefore, it is easy to identify situation (d), since this is the only one with positive ROESY peaks, and also to identify direct NOEs to rapidly exchanging water (b), since this is the only one with negative peaks in both. The difficult one to characterise is positive NOESY and negative ROESY, since this could arise in (a) or (c): unfortunately

Figure 6  Origin of intermolecular water-protein cross peaks in NOESY and ROESY spectra. In each case, the sign of the cross peak in NOESY and ROESY spectra is indicated, where + indicates a peak of the same phase as the diagonal. (A) Magnetisation transfer by direct NOE between the protons of a tightly bound (long residence time, likely buried) hydration water molecule and protein. The chemical exchange between the hydration water molecules and the bulk water leads to a single averaged water signal. (B) Similar to (A) except that the bound water has a short residence time and is therefore more likely to be on the surface. (C) Exchange-relayed NOE. The NOE between a non-labile and an exchangeable proton (e.g., a hydroxyl proton) of the protein appears at the water chemical shift, because of rapid exchange between the labile proton and the water. (D) Direct chemical exchange between an exchangeable proton of the proton and the water proton. Modified from Ref. 153.
this is also the most interesting, since if (a) it corresponds to long-residence time water. The only way to identify this safely is to be able to eliminate case (c) by looking at the protein structure and discounting any cases where there is a potential exchanging proton close by. The interpretation of long residence and short residence depends on the model used, and the common interpretation is that ‘long residence’ corresponds to waters with a residence time longer than about 1 ns.153

Using this theoretical basis, the hydration of biological molecules has been studied, and a large number of NOES to waters have been observed. A small number of water molecules, most of which are completely buried within the protein interior and are also observed in crystal structures, were shown to have residence times longer than 1 ns, while most water molecules were much more mobile.153 Studies on protein–ligand complexes often identified water molecules bound in the interface, many of these having long residence times.154,155 A variety of methods have been used to probe water residence times in more detail. However, three important papers were published by Halle and colleagues in 2003 and 2004,156–158 demonstrating that the residence times for water obtained from NOESY/ROESY ratios are very dependent on the model used, and that unfortunately the standard model used up to that time was not adequate, because cross-relaxation to bulk water (by contrast to individual waters in the hydration layer) turns out to be very significant. More recent work by others supports this general conclusion.159 The main consequence of this is that although the lifetimes derived for the small number of tightly bound waters were reasonable, lifetimes for more weakly bound waters were not, and in fact the large majority of hydration waters have dynamics retarded by a factor of less than 2 as compared to bulk water. Moreover, the relatively subtle differences in NOE/ROE ratio interpreted as differences in residence time are mainly due to different exposure of the site to bulk water. This result appears to invalidate many of the studies carried out on macromolecular hydration. There is a hydration shell of approximately 2 Å with a viscosity approximately two times greater than bulk water.156 The conclusion is thus that water lifetime depends mainly on geometric factors, with water being more retarded the deeper and more isolated it is within the protein.

Despite the rather limited range of information available on water residence times, the large number of publications on protein–water interactions had the effect of stimulating interest in pulse sequences and experiments for studying water interactions. One outcome of this was the experiment known as Water-LOGSY,160 which has proved extremely useful for screening ligand binding. The idea is that when a ligand binds to a protein (in a productive way, i.e., in a well-formed binding site) then it traps or retards one or more water molecules in the ligand–protein interface, such that there is at least one water molecule with a residence time longer than about 1 ns. On saturation of the water signal, there is a negative NOE to the ligand. By contrast, non-interacting ligands have positive NOEs. The difference is immediately obvious, since all the components have negative signal except for interacting molecules, which have positive signals, and the experiment is very sensitive, allowing experiments to be conducted with protein concentrations down to a few μM.
6. NOES INVOLVING NUCLEI OTHER THAN $^1\text{H}$

6.1. NOEs involving fluorine

$^{19}\text{F}$ has a gyromagnetic ratio close to that of $^1\text{H}$. This means the NOE between $^{19}\text{F}$ and $^1\text{H}$ is large, even for molecules with long correlation time such as proteins (behaving much like the $^1\text{H}$–$^1\text{H}$ NOE, going from positive to negative as the correlation time increases), and the cross-relaxation rate between $^{19}\text{F}$ and $^1\text{H}$ is relatively fast, though nothing like as fast as that between $^1\text{H}$ and $^1\text{H}$. Consequently, NOEs from $^{19}\text{F}$ to $^1\text{H}$ in proteins build up rapidly, but dissipate through the protein by spin diffusion even more rapidly. Therefore for detection of $^{19}\text{F}$ NOEs it may be necessary to perdeuterate the protein, leaving only $^{15}\text{N}$–$^1\text{H}$ protons. An elegant application of this method was described\(^\text{161}\) in which MgF\(_3\) (a transition state analog of a transferring phosphate group) was bound into the active site of a perdeuterated and $^{15}\text{N}$-labelled phosphoryl transferase, giving three fluorine signals. Saturation of each fluorine in turn gave NOEs to HN, detected by a difference HSQC experiment, thereby identifying the neighbours of each fluorine.

When fluorine is present in the solvent (e.g., as trifluoroethanol) then the concentration of fluorine is high enough that NOEs from fluorine to protein are large enough to be seen even without deuteration of the protein, for example by 1D gradient-detected NOE\(^\text{162}\) or 2D methods.\(^\text{163}\) As discussed in section 5, the NOE from fluorine to proton can be either positive or negative depending on the lifetime of bound trifluoroethanol, and both cases were observed.

NOEs from $^{19}\text{F}$ to $^{19}\text{F}$ can also be observed, and do not suffer from spin diffusion as badly. It is therefore possible to label proteins site-specifically with fluorines and detect close contacts using $^{19}\text{F}$–$^{19}\text{F}$ NOEs. This was done using rhodopsin in micelles, with fluorine labelling achieved by attachment of trifluoroethyl thio groups to genetically engineered cysteines, thereby presenting evidence for the arrangement of the transmembrane helices.\(^\text{164}\)

6.2. Dynamic nuclear polarisation

The main reason for the low sensitivity of NMR is that the population difference between the energy levels is small, implying that the magnetisation corresponding to the transition between the energy levels is also small. There are a variety of ways of hyperpolarising spins, vastly increasing the population difference, and therefore making the signal much bigger. This is a very useful way of increasing sensitivity; and the population difference can then be passed on to other spins via cross-relaxation, thereby producing potentially very large signals from neighbouring spins.

One way of doing this is known as Chemically Induced Dynamic Nuclear Polarisation (CIDNP) and involves irradiation of a flavin photosensitiser with light. This produces a radical, which polarises nearby aromatic systems such as tyrosines and tryptophans. This polarisation can then be passed on to
neighbouring spins by cross-relaxation, giving large NOEs.\textsuperscript{165} It has been used recently to enhance signals and thereby provide real-time information on protein folding.\textsuperscript{166}

Another way of polarising nuclei is by microwave irradiation at very low temperature (of the order of 1 K). For study of proteins, the sample then has to be transferred rapidly to a protein solution and warmed up, before the polarisation can dissipate via relaxation. This does not usually provide enough time for cross-relaxation before the signal is lost, although the potential is certainly there.\textsuperscript{167}

Xenon is a spin-1/2 nucleus, with a chemical shift that changes significantly depending on its environment. More importantly, it can be hyperpolarised by optical pumping with a laser, thereby amplifying the magnetisation by a factor of up to $10^5$. In favourable cases this can be passed on to neighbouring protons by cross-relaxation,\textsuperscript{168} an experiment known as SPINOE.\textsuperscript{169} Therefore experiments have been carried out in which Xe is added to protein solutions. Obvious disadvantages to the method are the limited solubility of Xe in water, and the weak binding of Xe to proteins (mM in both cases), but the biggest problem turns out to be the rather long correlation time of Xe, which in practice makes the NOEs very small.\textsuperscript{170}

**ABBREVIATIONS**

- DHPC: dihexanoylphosphatidylcholine
- NOE: nuclear Overhauser effect
- RDC: residual dipolar coupling
- rf: radiofrequency
- STD: saturation transfer difference
- trNOE: transferred NOE

**ACKNOWLEDGMENT**

I thank David Neuhaus for careful reading of the manuscript.

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