The Nuclear Overhauser Effect in NMR
Structure and Dynamics Analysis

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Content

The Nuclear Overhauser Enhancement or Effect (NOE) is the most important measure in liquid-state NMR for the characterization of the structure and dynamics of biomacromolecules. In this lecture, theoretical and practical aspects of the NOE are presented: A derivation of the NOE from relaxation theory, spin diffusion, traditional experiments to measure the NOE, exact measurements of the NOE (eNOE), transferred NOE (trNOE), the use of distance restraints from NOEs in structure calculation, and protocols for structural ensemble calculation in CYANA.

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1. A brief history of the NOE

The nuclear Overhauser enhancement or effect (NOE) is the cross-relaxation of spin polarization from one spin to another induced by dipole-dipole interaction. Because its amplitude depends on the separation of the two spins, it can be used to measure the distance between them. The NOE has arguably become the most important phenomenon in nuclear magnetic resonance (NMR) spectroscopy with biomolecules.

![Cartoon depiction of the Nuclear Overhauser Effect](image)

**Figure 1.1.** Cartoon depiction of the Nuclear Overhauser Effect. Two nuclei are shown in red, their magnetic moments in blue, the induced field lines of the left nucleus in black, the internuclear distance in green, and the polarizing magnetic field in grey, respectively.
In the following, a brief list of the milestones in the NOE research is presented. Years refer to the year of the publication rather than the year of reception of the manuscripts.

1953 The general Overhauser effect is **first predicted** by American physicist Albert Overhauser: He observed that saturation of the conduction electron spin resonance by microwave irradiation causes a polarization of nuclear spins of certain metals [1.1]; the extent of polarization is given by the ratio of the gyromagnetic ratios of the two spins; since these groundbreaking discoveries, the application of the NOEs branched into many dedicated fields

1953 The general Overhauser effect is **first demonstrated experimentally** by American physicists Tom R. Carver and Charles Pence Slichter: They observed an enhancement of the nuclear resonance in metallic lithium upon electron saturation [1.2]; the polarizing field was 30.3 Gauss (3.03 mTesla)

![Figure 1.2: First observation of the NOE. Top line: Lithium signal (lost in noise); middle line: Lithium signal enhanced by electron saturation. Taken from reference [1.2].](image)

1954 The general Overhauser effect is **predicted for non-metals** by Swiss-American physicist Felix Bloch (Nobel prize in physics 1952) [1.3]

1955 French physicist Ionel Solomon formulates the **Solomon equations** and verifies them experimentally by **measuring the nuclear Overhauser effect**: These differential
equations describe the magnetization changes of two spins under dipolar interaction [1.4]; although later extended, the simple theoretical description remains the most practical formulation of the NOE; the sample was hydrofluoric acid HF (hydrogen-fluor spin system)

1956 The NOE between hydrogens is observed: John Wertz, P.L. Jain and R.L. Batdorf observed the effect for methanol (CH₃OH, AX₃ spin system) by the field sweeping technique [1.5]

1962 The NOE is and experimentally demonstrated and rationalized for hydrogens: Weston Anderson and British chemist Raymond Freeman used double irradiation techniques on acetaldehyde (CH₃CHO, AX₃ spin system) [1.6]

1963 The NOE is used for assignment of NMR spectra: Reinhold Kaiser used the double irradiation technique to assign multiplets of AB₂C and ABR₃X type spin systems [1.7]

1965 The use of the internuclear distance dependence of the NOE for resonance assignment and conformational studies of small molecules in solution: Because the NOE occurs through space rather than through chemical bonds, Frank A. Anet and Anthony J.R. Bourn proposed the NOE as a probe for the proximity of atoms to each other [1.8]

1970 Experimental demonstration of direct correlation between NOE and distance: R.A. Bell and J.K. Saunders used the steady-state NOE to derive interproton distances in small organic molecules [1.9]; they obtained a near-perfect correlation between NOE enhancement and theoretical distances for well-isolated proton-proton and proton-methyl groups

Observation that the NOE also transfers magnetization between molecules (trNOE): Aksel A. Bothner-By and co-workers provided an equation describing the NOE in exchanging systems and determined fractions of bound ligands [1.11]

trNOE as a tool to study bound ligand conformations: J.P. Albrand, B. Birdsall, J. Feeney, G.C.K. Roberts and A.S.V. Burgen demonstrated the proximity of specific protons in trimethoprim and NADP⁺ in their complexes with dihydrofolate reductase by detection of signals from the free forms [1.12]

detected by 2D spectra [1.13]; crucial step that would later allow protein and nucleic acid structure calculation from NOEs

1980 The first 2D NOESY is recorded: Anil Kumar, Richard R. Ernst and Kurt Wüthrich (Nobel prize in chemistry 2002) recorded a proton-proton 2D NOESY of basic pancreatic trypsin inhibitor (BPTI) on a 360 MHz magnet [1.14]

![Figure 1.4: 2D NOESY of BPTI. Taken from reference [1.14]](image.png)

1980 The Tropp model is introduced: James Tropp developed the theoretical treatment of the NOE of a spin pair separated by a fluctuating distance [1.15]

1981 Transient NOE buildups in 2D NOESY are recorded: Based on these measurements, Anil Kumar, Gerhard Wagner, Richard R. Ernst and Kurt Wüthrich proposed ‘investigations of three-diensional structures of biopolymers’ [1.16]

1984 2D rotating-frame Overhauser effect spectroscopy (ROESY) is introduced: Aksel A. Bothner-By, Richard L. Stephens, Ju-mee Lee, Christopher D. Warren and R. W. Jeanloz determined the structure of a tetrasaccharide by observation of cross-relaxation between
magnetization spin-locked along a transverse axis [1.17]; ROESY yields cross peak when those in NOESY vanish due to tumbling times in the ‘blind range’

**1984 Full-matrix approach** is proposed for distance extraction: Joe W. Keepers and Thomas L. James diagonalized the relaxation matrix containing the cross-relaxation rate constants to analyze cross peak buildups [1.18]; two years prior, a similar approach has been proposed by C.M. Dobson, E.T. Olejniczak, F.M. Poulsen and R.G. Ratcliffe using measurement of time-dependent NOE enhancement under selective irradiation [1.19]

**1984/5** The first **NMR structures** of globular proteins are solved: In 1984, Alexander Arseniev, Vladimir Kondakov, Vladimir Maiorov and Vladimir Bystrov published an α/β-type structure of the 35-residue scorpion insectotoxin I5A using a method being under development in the Wüthrich laboratory and guessing the 4 most plausible disulfide bridges [1.20]; one year later, Michael P. Williamson, Timothy F. Havel and Kurt Wüthrich determined the structure of 57-residue bull seminal proteinase inhibitor IIA (BUSI IIA) containing an antiparallel β sheet, a short 310 helix, a regular α helix and three disulfide bridges [1.21]; they obtained 202 distance restraints from 2D NOESY at a concentration of 16 mM and a 500 MHz field; as early as in 1981, ‘preliminary’ structures of the segment comprising residues 19-27 of the 29-residue peptide glucagon bound to dodecylphosphocholine micelles have been determined by a series of 1D ‘driven Overhauser effect’ spectra and a distances geometry (DG) algorithm in the Wüthrich laboratory [1.22]
Figure 1.5. Original schematic representations of the backbone traces of IaA (left) and BUSI IIA (right) as shown in references 1.20 and 1.21.

1988 **Heteronucleus-resolved 3D NOESY** is introduced: Stephen W. Fesik and Erik R.P. Zuiderweg designed three-dimensional HMQC-NOESY, which simplifies the spectra substantially [1.23]; this development is the starting point for structure determination of $^{13}$C,$^{15}$N-labeled proteins of sizes up to ca. 20 kDa

1989 Structures from **averaged NOE-derived distances**: A.E. Torda, R.M. Scheek and Wilfred F. Van Gunsteren used time-dependent distance restraints in molecular dynamics simulations [1.24]

1990 **4D NOESY** is introduced: Lewis E. Kay, G. Marius Clore, Ad Bax and Angela M. Gronenborn demonstrate a substantial improvement of the resolution of spectra of interleukin-1 beta, a 153-residue protein [1.25]; the 4D $[^{13}$C,$^{1}$H]-HMQC-NOESY-$[^{15}$N,$^{1}$H]-HMQC opened the way for a succession of improvements of 4D NOESY that has been pursued up to this day; $[^{13}$C,$^{15}$N]-labeled proteins of sizes up to ca. 40 kDa can be analyzed

1995 Arguably the first **multi-state structure** calculation from **exact NOEs**: Initial NOE
buildup curves were used for an ensemble calculation of the peptide YQNPDGSQA [1.26]

**1995** Introduction of **ambiguous distances restraints**: Michael Nilges proposed to use NOE-derived distance restraints that are not unambiguously assigned in structure calculation [1.27]

**1997** NOESY with highly **deuterated, methyl-reprotonated** proteins: Kevin H. Gardner, Michael K. Rosen and Lewis E. Kay obtained global folds of proteins using this novel labeling scheme that allows application to large proteins [1.28]

**Motivation/goal of this lecture**

The subjects of this lecture are mostly i) the transient nuclear Overhauser effect buildup as observed in two proton dimensions and ii) the transferred NOE. The collection of many exact transient NOEs (eNOEs) ultimately allowed our laboratory to calculate structural ensembles of proteins consisting of multiple states [1.29]. The transferred NOE (trNOE) is used to study protein/ligand interaction.

Standard structure determination by NMR spectroscopy makes use of a large number of experimentally accessible NOE rates – typically up to 20 per residue in small proteins. Since the NOE rate is inversely proportional to the sixth power of the distance between the two dipolar interacting spins, the strength of the NOE lies in the supply of a large number of through-space distance restraints. Usually, these restraints are employed in a semi-quantitative manner at most because the measurement of NOEs is flawed by mobility, spin diffusion, low signal-to-noise ratio and technical limitations.
We have demonstrated that it is possible to obtain exact proton/proton NOEs (eNOEs) in protein samples [1.30]. To this purpose, we measured NOE buildups as a function of the NOESY mixing time and converted the NOEs into precise distances. For example, distances up to 5 Å obtained from a perdeuterated ubiquitin sample have an experimental random error of only ≈ 0.07 Å. Since the eNOE is a time- and ensemble-averaged observable, it contains both structural and dynamical information. The collection of potentially thousands of eNOEs throughout a biomacromolecule serves as an excellent probe towards a more complete representation of both its structure and dynamics. In addition, the NOE is among the few observables that are measurable even for high molecular weight systems such as large proteins, protein complexes, or membrane proteins substituted in membrane-mimicking environments.

We also established a new CYANA protocol, which calculates multiple-state ensembles of structures in which the conformational restraints are required to be fulfilled on average over all members of the ensemble rather than for each individual conformer. Such ensembles allow a broad characterization of macromolecules, for example, they may indicate the presence of correlated motion.

The goal of this lecture is that the participant gains understanding of the underlying principles of the NOE, gets familiar with the pulse sequences used to measure the NOE, learns to extract exact distance limits from NOE and to calculate multi-state bundles of a protein, and to conduct trNOE experiments.
2. Theoretical background

2.1. Classical derivation of the NOE

Most textbooks introduce the nuclear Overhauser effect and the differential equations describing the NOESY process (the Solomon equations) classically. It is recommended to study this approach to obtain initial insight.

We assume a system consisting of two spins of value $\frac{1}{2}$, $I$ and $S$. Their spin components along the $z$-axis (parallel to the polarizing field) are ‘up’ or ‘down’, designated by their respective eigenstates $|\alpha\rangle$ and $|\beta\rangle$. The four eigenstates of the system are $|\alpha\alpha\rangle$, $|\alpha\beta\rangle$, $|\beta\alpha\rangle$ and $|\beta\beta\rangle$ as indicated in Figure 2.1. Their population numbers are $N_{\alpha\alpha}$, $N_{\alpha\beta}$, $N_{\beta\alpha}$ and $N_{\beta\beta}$. The transition probabilities per unit time between the states are $W_{I\alpha}$, $W_{I\beta}$, $W_{S\alpha}$ and $W_{S\beta}$. These transitions are essentially energy exchanges among the spins of the systems. Together with energy exchange with the solution (lattice, continuum due the many degrees of freedom), they are responsible for relaxation of a spin system in NMR. Spontaneous emission or absorption is not relevant. In order to mediate an energy transfer, the motion in the lattice that gains or loses the energy from the nuclear spin transition must itself cause a fluctuating magnetic field at the spin. The frequency of this motion must correspond to the frequency of the transition. Therefore, the mechanism depends on time scales.
Figure 2.1. Energy-level diagram of a two-spin system. The transition probabilities between the eigenstates are indicated by red, blue and purple arrows, for spin $I$, spin $S$ and double flips, respectively. The populations are represented by the grey balls.

We construct the equations of temporal evolution of the population numbers by the following differential equations:

$$\begin{align*}
\frac{dN_{\alpha\alpha}}{dt} &= -(W_{1I} + W_{1S} + W_{2IS}) N_{\alpha\alpha} + W_{1S} N_{\alpha\beta} + W_{1I} N_{\beta\alpha} + W_{2IS} N_{\beta\beta} + \text{const} \\
\frac{dN_{\alpha\beta}}{dt} &= W_{1S} N_{\alpha\alpha} - (W_{0IS} + W_{1I} + W_{1S}) N_{\alpha\beta} + W_{0IS} N_{\beta\alpha} + W_{1I} N_{\beta\beta} + \text{const} \\
\frac{dN_{\beta\alpha}}{dt} &= W_{1I} N_{\alpha\alpha} + W_{0IS} N_{\alpha\beta} - (W_{0IS} + W_{1I} + W_{1S}) N_{\beta\alpha} + W_{1S} N_{\beta\beta} + \text{const} \\
\frac{dN_{\beta\beta}}{dt} &= W_{2IS} N_{\alpha\alpha} + W_{1I} N_{\alpha\beta} + W_{1S} N_{\beta\alpha} - (W_{1I} + W_{1S} + W_{2IS}) N_{\beta\beta} + \text{const}
\end{align*}$$

(2.1.1-4)

The observable quantities are the macroscopic magnetic moments $\langle I_z \rangle$ and $\langle S_z \rangle$ (‘magnetization’), which are proportional to the sum over the magnetic moments of all spins in
the sample. It follows that the resulting magnetization is proportional to the difference of the population numbers:

\[
const'\langle I_z \rangle = \left( N_{\alpha\alpha} + N_{\alpha\beta} \right) - \left( N_{\beta\alpha} + N_{\beta\beta} \right)
\]

\[
const'\langle S_z \rangle = \left( N_{\alpha\alpha} + N_{\beta\alpha} \right) - \left( N_{\alpha\beta} + N_{\beta\beta} \right)
\]  

(2.2.1-2)

Inserting equation 2.2 into 2.1 gives (exercise 1):

\[
\frac{d\langle I_z \rangle}{dt} = -(W_{0IS} + 2W_{1IS} + W_{2IS})\langle I_z \rangle - (W_{2IS} - W_{0IS})\langle S_z \rangle + const
\]

\[
\frac{d\langle S_z \rangle}{dt} = -(W_{0IS} + 2W_{1IS} + W_{2IS})\langle S_z \rangle - (W_{2IS} - W_{0IS})\langle I_z \rangle + const
\]  

(2.3.1-2)

Using explicit values for the constants, we choose the equilibrium values of the magnetizations:

\[
\frac{d\langle I_z \rangle}{dt} = -(W_{0IS} + 2W_{1IS} + W_{2IS})\langle I_z - I_{z,0} \rangle - (W_{2IS} - W_{0IS})\langle S_z - S_{z,0} \rangle
\]

\[
\frac{d\langle S_z \rangle}{dt} = -(W_{0IS} + 2W_{1IS} + W_{2IS})\langle S_z - S_{z,0} \rangle - (W_{2IS} - W_{0IS})\langle I_z - I_{z,0} \rangle
\]  

(2.4.1-2)

Writing equation 2.4 in the form of extended Bloch equations for the longitudinal magnetization:

\[
\frac{d\langle I_z \rangle}{dt} = -\rho_I \langle I_z - I_{z,0} \rangle - \sigma_{IS} \langle S_z - S_{z,0} \rangle
\]

\[
\frac{d\langle S_z \rangle}{dt} = -\rho_S \langle S_z - S_{z,0} \rangle - \sigma_{IS} \langle I_z - I_{z,0} \rangle
\]  

(2.5.1-2)

with

\[
\rho_I = W_{0IS} + 2W_{1IS} + W_{2IS}
\]

\[
\rho_S = W_{0IS} + 2W_{1IS} + W_{2IS}
\]

\[
\sigma_{IS} = -(W_{0IS} - W_{2IS})
\]  

(2.6.1-2)

These are the Solomon equations with \( \rho_{IS} \) being the autorelaxation rates of spin \( I \) and \( S \), and \( \sigma_{IS} \) the cross-relaxation rate.
2.1.1. Steady-state Overhauser effect

In the steady-state condition, the time derivative vanishes. From equation 2.5.1 we obtain

\[ 0 = -\rho_I \langle I_z - I_{z0} \rangle - \sigma_{IS} \langle S_z - S_{z0} \rangle \]  

(2.7)

If the \( |\alpha\rangle \) and \( |\beta\rangle \) populations of spin S are equalized by irradiation (\( S_z = 0 \)), S is said to be saturated. We obtain

\[ \langle I_z \rangle = I_{z0} + \frac{\sigma_{IS}}{\rho^I} S_{z0} \]  

(2.8.1)

\[ \frac{\langle I_z \rangle}{I_{z0}} = 1 + \frac{\sigma_{IS} S_{z0}}{\rho I} = 1 + \frac{\sigma_{IS} \gamma^I}{\rho I} \equiv 1 + \eta_{IS} \]  

(2.8.2)

This effect is called the ‘steady-state NOE’. At present, its most popular use is the heteronuclear NOE measurement in order to determine order parameters of H-N or H-C spin pairs with fixed distances. Many initial attempts at proton-proton distance determination made use of buildup measurements of the intensity of spin I, because spin diffusion renders the steady-state approach impractical.

2.1.2. Transient Overhauser effect

Assume that \( \langle S_z \rangle \) deviates from the equilibrium \( S_{z0} \) (e.g. after a selective polarization inversion pulse, \( \langle S_z \rangle = -S_{z0} \)). The initial conditions are

\[ \langle I_z - I_{z0} \rangle_{t=0} = 0 \]

\[ \langle S_z - S_{z0} \rangle_{t=0} = S_z \]  

(2.9.1-2)
To get first insight, we assume that \( \rho_I = \rho_S \). We obtain a simple solution for equation 2.5 (exercise 2):

\[
\langle I_z \rangle - I_{z,0} = \frac{1}{2} S [ e^{-(\rho+\sigma_I)t} - e^{-(\rho-\sigma_I)t} ]
\]

\[
\langle S_z \rangle - S_{z,0} = \frac{1}{2} S [ e^{-(\rho+\sigma_S)t} + e^{-(\rho-\sigma_S)t} ]
\]

(2.10.1-2)

If \( \langle S_z \rangle \) deviates from the equilibrium \( S_{z,0} \), the population difference of spin \( I \) is altered. This effect is called ‘transient NOE’ and is the mechanism behind NOESY.

2.1.3. The cross-relaxation rate

To obtain an expression for the cross-relaxation rate \( \sigma_{IS} \), the transition probabilities \( W_{1I} \), \( W_{1S} \), \( W_{0IS} \) and \( W_{2IS} \) have to be evaluated. This is done by applying Fermi’s golden rule to the Hamiltonian of the dipolar interaction, which is based on quantum mechanics. We will obtain the result in the semi-classical derivation of the NOE and therefore directly give the result here for a rigid molecule:

\[
\sigma_{IS} = \left( \frac{\mu_0}{4\pi} \right)^2 \frac{\gamma^4 \hbar^2}{10} \frac{\tau_c}{r_{IS}^6} \left[ -1 + \frac{6}{1 + 4\omega_0^2 \tau_c^2} \right]
\]

(2.11)

where \( I \) and \( S \) are assumed to be the same atom type (typically protons). The overall tumbling time is \( \tau_c \) and the larmor frequency is \( \omega_0 \). All other constants have the common meaning (see later).
Although not calculated in the seminal paper by Solomon, the key aspect of the expression is the $r^{-6}$ dependency on the spatial spin separation, which enables the distance measurements. The steady-state NOE enhancement factor is

$$\eta_{IS} = \frac{1}{\rho_i} \left( \frac{\mu_0}{4\pi} \right)^2 \gamma^4 h^2 \frac{\tau_c}{10} \frac{r_{IS}^6}{r_{IS}^6 - 1 + 6 \frac{1}{1 + 4\omega_0^2 \tau_c^2}} $$

(2.12)

2.2. Semi-classical derivation of the NOE

Here the derivation is based on the quantum-chemical approach in combination with a classical treatment of the lattice. Only this approach provides the more subtle mechanisms that are also active in NOESY. A detailed understanding of these effects and the conditions for their neglect is important in the view of exact quantitative evaluation of NOEs.

2.2.1. The Master equation

The time evolution of a spin system is described by the Liouville-von Neumann equation.

$$\frac{d}{dt} \rho(t) = -i \left[ H_0 + H_1(t), \rho(t) \right] $$

(2.13)

$\rho(t)$ is the density operator, $H_0$ is the stationary Hamiltonian and $H_1(t)$ is the stochastic Hamiltonian which couples the spins to the lattice. The equation may be expressed in the interaction frame by transformation of any operator $K$:

$$K^{\text{int}}(t) = e^{iH_0t}Ke^{-iH_0t} $$

(2.14)
Quantum mechanical and classical treatments of the nuclear spin system and the lattice, respectively, have proven most practical for NMR descriptions. The semi-classical Master equation in the interaction frame is a solution to the Liouville-von Neumann equation:

$$\frac{d}{dt} \langle \rho_{in}^\text{int}(t) - \rho_0 \rangle = - \int_0^{\tau_{mix}} \left[ \left[H_1^\text{int}(t), \left[H_1^\text{int}(t-\tau), \rho_{in}^\text{int}(t) - \rho_0 \right] \right] \right] d\tau$$

(2.15)

As NMR experiments deal with a large number of molecules, ensemble averaging over all stochastic Hamiltonians is used. The equilibrium density operator $\rho_0$ is introduced ad hoc since in the semi-classical approach the Boltzmann equilibrium is not re-established. Additional assumptions used to obtain equation 2.15 may be looked up in the literature [2.1,2.2]. One common assumption, however, is not made here. The upper limit of the integral is usually replaced by infinity. In experiment, however, it is only carried out for the length of the mixing time $\tau_{mix}$ (typically 100 ms in a NOESY).

The stochastic Hamiltonian can be decomposed into products of spin operators given by irreducible tensor operators of rank 2, $T_m$, and stationary random functions given by second order spherical harmonics, $F_m$, where $m$ are coherence levels:

$$H_1(t) = \sum_{m=-2}^2 T_m F_m(t)$$

(2.16.1)

The conventions of reference [2.2] are used. Transformation of $H_1(t)$ into the interaction frame gives:

$$H_1^\text{int}(t) = \sum_{m,n} T_m^n F_m(t) e^{i\Delta_m^n}$$

(2.16.2)
The $T_m$ operators are split into contributions corresponding to the differences between the eigenfrequencies of $H_0$. More details are shown in equations A1-A10 in the Appendix. When $H^\text{int}_1(t)$ for all interactions $a$ are inserted into equation 2.15, it takes the form:

$$\frac{d}{dt}\langle \rho^{m}(t) - \rho_0 \rangle =$$

$$- \sum_{m,m',n,n',a,a'} (-1)^{m'} e^{i(\omega^{n,a}_m + \omega^{n',a'}_{m'})} \left[ T^{n',a'}_{m'}, \left[ T^n_{m}, \langle \rho^{\text{int}}(t) - \rho_0 \rangle \right] \right] \int_0^{t} \left\langle F^{n,a}_{-m}(t+\tau) F^{a}_{m}(t) \right\rangle e^{-i\omega^{n,a'}_{m'}\tau} d\tau$$

(2.17)

In the secular approximation only terms with $\omega^{n,a}_m \approx -\omega^{n',a'}_{m'}$ are retained. All other terms contain a rapidly oscillating component that averages them to zero. It can be shown that $m'$ can be replaced by $-m$. Back-transformation into the laboratory frame leads to the semi-classical Master equation for the expectation value of the operator $B$, $\langle B \rangle = \text{Trace}(B\rho)$:

$$\frac{d}{dt}\langle B \rangle(t) = \langle -i [B, H_0] \rangle(t) - \langle \dot{\Gamma}(B) \rangle(t) - \langle \dot{\Gamma}(B) \rangle_0$$

(2.18)

with the relaxation superoperator for non-degenerate transitions

$$\dot{\Gamma}(B) = \sum_{m,n,n',a,a'} (-1)^m J^{n,a}_{m} (\omega^{n,a}_m) \left[ T^n_{m}, \left[ T^{n',a'}_{m'}, B \right] \right]$$

(2.19.1)

$n^{nd}$ are those $n'$ that fulfill $\omega^{n,a}_m \approx -\omega^{n',a'}_{-m}$. The relaxation superoperator for degenerate transitions is

$$\dot{\Gamma}(B) = \sum_{m,n,n',a,a'} (-1)^m J^{n,a}_{m} (\omega^{n,a}_m) \left[ T^n_{m}, \left[ T^{n',a'}_{-m}, B \right] \right]$$

(2.19.2)

$n^{d}$ are those $n'$ that fulfill $\omega^{n,a}_m \approx -\omega^{n',a'}_{-m}$ with additional pathways from the fact that $\omega_i - \omega_j = 0$. 

23
$J_m^{aa'}(\omega)$ is the spectral density function which carries the information on the fluctuating angular orientations and distances of the interaction vectors (vide infra).

$$J_m^{aa'}(\omega) = \int_0^{\tau_{\text{eq}}} \left\langle F_m^{aa'}(t+\tau)F_m^{aa}(t) \right\rangle \cos(\omega \tau) d\tau \tag{2.20}$$

In a strict sense, $\cos(\omega \tau)$ must be replaced by $e^{-i\omega \tau} = \cos(\omega \tau) - i \sin(\omega \tau)$. However, the second term causes a small frequency shift and does not affect the relaxation behavior. As this effect is not relevant for the current work, the simplification is justified.

A convenient way to use the Master equation is to choose an (orthogonal) operator basis spanning the complete relevant Liouville subspace. The expectation values of these operators can be arranged in vector form $\langle \vec{b} \rangle$, such that the Master equation is written in matrix form:

$$\frac{d}{dt} \langle \vec{b} \rangle(t) = A \langle \vec{b} \rangle(t) - R \left( \langle \vec{b} \rangle(t) - \langle \vec{b} \rangle_0 \right) \tag{2.21}$$

$R$ is the relaxation matrix, and $A$ is the matrix describing the unperturbed motion of the spin system.

The relevant stationary and stochastic Hamiltonians for a $N$-proton system are:

$$H_0^N = -\gamma \vec{B}_0 \sum_{i=1}^{N} (1 - \Omega_i^{\text{CSI}}) \vec{I}_i \tag{2.22.1}$$

$$H_1^N(t) = \frac{\mu_0}{4\pi} \sum_{i<j}^{N} \frac{\gamma^2 \hbar}{r_{ij}^3(t)} \left( \vec{I}_i \vec{J}_j - \frac{3}{r_{ij}^3(t)} \left( \vec{I}_i \vec{J}_j(t) \right) \vec{I}_j \vec{J}_i(t) \right) \tag{2.22.2}$$

$\gamma$ is the gyromagnetic ratio of the proton, $\vec{B}_0$ the polarizing field vector, $\Omega_i^{\text{CSI}}$ is the isotropic part of the chemical shielding tensor of nucleus $i$ (referred to as CSI tensors) in the laboratory frame,
2.2.1. Non-degenerate transitions

Inspection of the Redfield Kite of the relaxation matrix $R$ shows that in the absence of degenerate transitions (that is separated by at least the linewidth), cross relaxation is only allowed between longitudinal operators. In the ideal case, the pulse sequence prior to the NOESY mixing period creates only longitudinal magnetization away from the (macroscopic) Boltzmann equilibrium magnetization. Any residual transverse magnetization is assumed to be dephased by a gradient and not considered further. The initial magnetization vector $\langle \vec{b}_0 \rangle$ contains only nonzero elements for the polarization operators (longitudinal single-spin order) $I_{i,x}$. As a consequence, zero-, single- and higher-quantum coherences are never created by evolution of longitudinal terms during the mixing time in a NOESY experiment. The complete Master equation for an $N$ spin-$\frac{1}{2}$ system can now be formulated in the subspace spanned by the population operators. $\langle \vec{b} \rangle$ is then built by (Ernst notation):
\[
\beta = \frac{1}{2} \prod_{i=1}^{N} (2I_{i,z})^\alpha_i
\]  

(2.23)

\(\alpha\) is a vector containing \(N\) elements \(\alpha_i\) which are either 0 or 1 \((1 \leq i \leq N)\). In total, \(2^N-1\) operators can be generated (the null vector which generates the identity operator is not included). Longitudinal multi-spin order terms are 0 at the outset of the mixing time and are subsequently created only for odd number orders and with decreasing efficiency as the order increases. For example, direct transfer from single-spin order can only occur to three-spin order populations. In the following, the explicit solution of the relaxation matrix is shown if only longitudinal single- and three-spin orders are considered. This subspace is sufficient to demonstrate all types of relaxation-matrix elements. Extension to higher spin orders is straightforward. The calculation of the matrix elements is presented in detail in the Appendix.

\[
\begin{pmatrix}
\langle I_{1,z} \rangle - I_{1,0} \\
\langle I_{2,z} \rangle - I_{2,0} \\
\langle I_{3,z} \rangle - I_{3,0} \\
\ldots \\
\langle 4I_{N-2,z} \rangle - I_{N,0} \\
\langle 4I_{N-1,z} \rangle - I_{N,0} \\
\langle 4I_{N,z} \rangle - I_{N,0} \\
\end{pmatrix} =
\begin{pmatrix}
\rho_1 & \sigma_{12} & \sigma_{13} & \ldots & \sigma_{1N} & \eta_{1213} & \eta_{1214} & \ldots & 0 \\
\sigma_{12} & \rho_2 & \sigma_{23} & \sigma_{2N} & \eta_{12213} & \eta_{12214} & \ldots & 0 \\
\sigma_{13} & \sigma_{23} & \rho_3 & \sigma_{3N} & \eta_{13213} & \eta_{13214} & \ldots & 0 \\
\ldots & \ldots & \ldots & \ldots & \ldots & \ldots & \ldots & \ldots \\
0 & 0 & 0 & \ldots & \eta_{NN-2NN-1} & 0 & 0 & \ldots & \rho_{NN-2NN-1} \\
\ldots & \ldots & \ldots & \ldots & \ldots & \ldots & \ldots & \ldots & \ldots \\
\ldots & \ldots & \ldots & \ldots & \ldots & \ldots & \ldots & \ldots & \ldots \\
\end{pmatrix}
\]

(2.24)

\(\rho_i\) and \(\rho_{ijk}\) are the autorelaxation rate constants of \(I_i\) and \(4I_{i,z}I_{j,z}I_{k,z}\), respectively. \(\sigma_{ij}\) is the cross-relaxation rate constant between spins \(I_i\) and \(I_j\) (sometimes referred to as the ‘NOE rate’). \(\eta_{ij}^{DD}\) is the longitudinal cross-correlated relaxation rate constant between two dipolar interactions. The rate constants contain the following contributions:
\[ \rho_i = \left( \sum_{j=1, j \neq i}^{N} R_{ij}^D \right) + R_{ij}^{\text{leak}} \]  
(2.25.1)

\[ \rho_{ijk} = \left( \sum_{l=1, l \neq i, j, k}^{N} R_{ij}^D \right) + \left( \sum_{l=1, l \neq i, j, k}^{N} R_{jl}^D \right) + \left( \sum_{l=1, l \neq i, j, k}^{N} R_{kl}^D \right) + R_{ij}^{\text{leak}} \]  
(2.25.2)

\[ \sigma_j = \left( \frac{\mu_0}{4\pi} \right)^2 \frac{\gamma^4 h^2}{10} \frac{1}{(r_{ij}^{\text{rigid}})^6} \left[ -J_{ij} (\omega_i - \omega_j) + 6J_{ij} (\omega_i + \omega_j) \right] \]  
(2.25.3)

\[ \eta_{ijk}^{D/D} = \frac{3}{5} \left( \frac{\mu_0}{4\pi} \right)^2 \frac{\gamma^4 h^2}{(r_{ij}^{\text{rigid}})^3 (r_{jk}^{\text{rigid}})^3} J_{ij} (\omega_i) \]  
(2.25.4)

with

\[ R_{ij}^D = \left( \frac{\mu_0}{4\pi} \right)^2 \frac{\gamma^4 h^2}{10} \frac{1}{(r_{ij}^{\text{rigid}})^6} \left[ J_{ij} (\omega_i - \omega_j) + 3J_{ij} (\omega_i) + 6J_{ij} (\omega_i + \omega_j) \right] \]  
(2.26)

\( R_{ij}^{\text{leak}} \) and \( R_{ijk}^{\text{leak}} \) are the leakage rates caused by additional relaxation processes such as chemical shift anisotropy (CSA), some additional dipolar interaction that is not relevant here, interaction with the solvent, paramagnetic reagents, NMR-active heteronuclei, etc. \( r_{ij}^{\text{rigid}} \) is the internuclear \( i-j \) distance in a hypothetically rigid structure. \( J(\omega) \) is the spectral density function at spectral frequency \( \omega \) as defined in equation 2.20. It depends on the exact nature of the vectors connecting spins \( I_i, I_j, \) and \( I_k \), and is described below. It is noted that the cross-correlation terms usually contain an additional factor \( P_z (\cos \theta) \), which describes the angle \( \theta \) between the two interaction axes. Here this dependence is included in the spectral density function. In doing so, the assumption of isotropic molecular tumbling is released.
2.2.2.2. Like spins

If the transitions are degenerate, diagonal and cross peaks appear at identical frequencies and can obviously not be evaluated separately in a spectrum. They may still be used by interpreting the sum of the single peaks. The relaxation rate constants are now calculated from equation 2.19.2 rather than from 2.19.1. One way to treat them is to allocate an individual magnetization in \( \langle \mathbf{b} \rangle \) to each proton and include the corresponding relaxation rates for degenerate transitions. In addition, exchange rates between the protons can also be included (vide infra).

However, the subspace chosen here is not sufficient to exactly express the relaxation of magnetically equivalent spins. It is convenient to formally treat a group of \( N \) spins like one spin with \( N \)-fold spectral intensity. This approach is most prominently applied to methyl groups and aromatic protons. The magnetization is written as

\[
I_{i,z} = \sum_{\kappa=1}^{N} I_{\kappa,z}
\]

\( i \) is a pseudo proton number in vector form including the protons \( i_1 \) to \( i_N \). A corresponding spectral peak is \( N \) times more intense than one expected from a single proton.

The cross relaxation rate constant between \( N_i \) and \( N_j \) magnetically equivalent spins is

\[
\sigma_{ij} = \sum_{\kappa=1}^{N_i} \sum_{\lambda=1}^{N_j} \frac{1}{N_j} \frac{1}{N_i} \left( \frac{\mu_0}{4\pi} \right) \frac{\gamma^4 h^2}{10} \left( \frac{r_{\text{agg}}^{-6}}{} \right) \left[ -J_{i,k} \omega_i - J_{j,k} \omega_j + 6J_{i,k} \left( \omega_i + \omega_j \right) \right]
\]

\( \sigma_{ij} \) is \( N_i \) times larger than expected for a single transfer pathway \( \sigma_{i,k} \). The summation can be absorbed into the spectral density functions.
\[ \sigma_{ij} = \left( \frac{\mu_0}{4\pi} \right)^2 \gamma^4 \hbar^2 \frac{N_i}{10} \frac{1}{(r_{\text{rigid}}^i j)^6} \left[ -J_y (\omega_i - \omega_j) + 6J_y (\omega_i + \omega_j) \right] \] (2.29)

The distance \( r_{\text{rigid}}^i j \) is the distance between the pseudoatoms representing the groups \( \tilde{i} \) and \( \tilde{j} \).

An additional complication arises from the lost symmetry of the relaxation matrix \( R \) as
\[ N_j \sigma_{ij} = N_i \sigma_{ji} \] and thus in general \( \sigma_{ij} \neq \sigma_{ji} \). A solution has been proposed for symmetrization [2.3].

2.2.2.3 Near-degenerate transitions

The situation is different if the peaks are barely resolved. Then the transitions may be near-degenerate. There are pathways to zero-quantum coherence between degenerate transitions mediated by cross-correlated relaxation (see Tables A4 and A5). This implies that the formulae for non-degenerate transitions can be used if the cross-correlated relaxation is sufficiently small.

As a rule-of-thumb, it has to be taken into account if \( \omega_m^i + \omega_m^j \approx \langle |H^\text{int}_1 (t)|\rangle \tau_c \) holds, where \( \tau_c \) is the effective molecular tumbling time (\textit{vide infra}). In practice, this is relevant for spectral frequencies separated by no more than 2 to 20 s\(^{-1}\). This range is typically less than the line width for macromolecules.

An additional complication arises if the spins are strongly scalar coupled, which can occur for protons if they have non- or near-degenerate transitions (for example, methylene protons). Then the basis chosen in equation 2.23 is no longer appropriate. Instead, the eigenfunctions of \( H_0 \) are linear combinations of the wavefunctions of the strongly coupled spins and the eigenvalues also depend on the scalar coupling constant.
2.2.3. The Solomon equations II

Further simplifications lead to the generalized form of the Solomon equations. Dipole/dipole cross-correlated relaxation has a rather small effect in NOESYs. Thus, there is no efficient transfer mechanism to populate two- and higher-spin orders. To a good approximation, the relevant NOESY process can be described by Solomon equations extended to an $N$-spin system, which are formulated in the reduced space spanned only by $N$ single-spin order operators:

$$\frac{d}{dt} \begin{pmatrix} \langle I_{1,z} \rangle - I_{1,0} \\ \langle I_{2,z} \rangle - I_{2,0} \\ \vdots \\ \langle I_{N,z} \rangle - I_{N,0} \end{pmatrix} = - \begin{pmatrix} \rho_1 & \sigma_{12} & \cdots & \sigma_{1N} \\ \sigma_{12} & \rho_2 & \cdots & \sigma_{2N} \\ \vdots & \vdots & \ddots & \vdots \\ \sigma_{1N} & \sigma_{2N} & \cdots & \rho_N \end{pmatrix} \begin{pmatrix} \langle I_{1,z} \rangle - I_{1,0} \\ \langle I_{2,z} \rangle - I_{2,0} \\ \vdots \\ \langle I_{N,z} \rangle - I_{N,0} \end{pmatrix}$$

(2.30)

These equations are usually employed to analyze NOESY spectra.

2.2.4. Conformational/chemical exchange

Conformational and chemical exchange cause modulation of the Hamiltonians by shifting the Larmor frequency through the frequencies associated with each sampled conformation/state (among other changes). If the exchange is fast ($k_{ex} \gg \Delta \omega_i$), the different components of the magnetization are not resolved in a spectrum. However, if the exchange is slow ($k_{ex} \ll \Delta \omega_i$), separate components are resolved. This effect is illustrated in Figure 2.2.
Figure 2.2. Peak shapes in one dimension under conformational/chemical exchange of a single spin $i$. The exchange rate $k_{\text{ex}}$ ranges from fast ($k_{\text{ex}} \gg 100$ kHz) to slow ($k_{\text{ex}} \ll 100$ kHz), and equal populations of two states are assumed. The narrow line at 0 kHz corresponds to a fast exchange, while the two lines at ±100 kHz correspond to a slow exchange, and reveal the two distinct electronic environments of the spin. The broad middle line corresponds to the crossover point where $k_{\text{ex}}$ is ca. 100 kHz.

The exchange process during the NOESY mixing time $\tau_{\text{mix}}$ is given by the longitudinal component of the McConnell equations [2.4]. A $N_{\text{ex}}$-site first-order exchange process in chemical equilibrium is assumed for spin $i$:

$$I_{i,r} \xrightarrow{k_{i,r}} I_{i,s} \xleftarrow{k_{i,s}} I_{i,z}$$  \hspace{1cm} (2.31)

$r$ and $s$ designate a pair of different states. The consequence is that each $I_{i,z}$ magnetization is split into $N_{\text{ex}}$ components $I_{i,r,z}$ in equation 2.30 and a modified relaxation matrix $R' = K + R$ is used, where $K$ includes elements describing the exchange rates. The resulting subequation is
\[
\frac{d}{dt} \begin{pmatrix}
\langle I_{i,z} \rangle - I_{i,0} \\
\langle I_{i,r,z} \rangle - I_{i,r,0} \\
\langle I_{i,N_{ex},z} \rangle - I_{i,N_{ex},0}
\end{pmatrix} = \\
\begin{pmatrix}
\rho_{i,1} + \sum_{s=2}^{N_{ex}} k_{i,rs} & -k_{i,r1} & \cdots & -k_{i,rN_{ex}} \\
\vdots & \ddots & \ddots & \vdots \\
-k_{i,1r} & \rho_{i,r} + \sum_{s=1 \neq r}^{N_{ex}} k_{i,rs} & \cdots & \rho_{i,N_{ex}} + \sum_{s=1}^{N_{ex}-1} k_{i,N_{ex}s} \\
\end{pmatrix}
\begin{pmatrix}
\langle I_{i,z} \rangle - I_{i,0} \\
\langle I_{i,r,z} \rangle - I_{i,r,0} \\
\langle I_{i,N_{ex},z} \rangle - I_{i,N_{ex},0}
\end{pmatrix}
\] (2.32)

If the initial magnetic perturbation is nonselective, the components \( I_{i,r,z} \) are proportional to the populations of the exchanging sites. This is illustrated in Figure 2.3.

To simplify the mathematical expressions, it is common to assume that the autorelaxation rate is the same for all \( r \).

The consequences of slow exchange for a NOESY spectrum are that corresponding peaks show up on the diagonal and the exchange process builds up cross peaks that are nearly indistinguishable from those induced by cross-relaxation. This type of dynamics cannot be described with the spectral density function.

Equation 2.32 is at the core of the transferred NOE (trNOE).
Figure 2.3. Peak lineshape in one dimension under conformational/chemical exchange of a single spin $i$. The two sites are not equally populated (2:1). The fast exchange peak appears at the barycenter of the slow exchange peak positions weighted by their populations.

2.2.5. The spectral density function

For ensemble-based structure calculations employing exact NOEs, it is important to understand the implications of the impact of the motional behavior of the molecule on the NOE. If a spin gives rise to a single peak, it is associated with a single spectral frequency. The complete information on motion which is accessible by the NOESY experiment is imprinted on the spectral density function. The spectral density function is obtained from equation 2.20:

$$J_m^{aa'}(\omega) = \int_0^{\tau_{\text{mix}}} C_{\text{mix}}^m(\tau) \cos(\omega \tau) \, d\tau$$  \hspace{1cm} (2.33)

with the correlation function
\[ C^{a'a'}(\tau) = \left\langle F^a_m(t+\tau)F^a_m(t) \right\rangle \]  
\[ (2.34) \]

As \( F \) is a *stationary* random function, its dependence on \( t \) can be replaced by an evaluation at 0 without loss of generality. The correlation function is independent of \( m \) and equation 2.34 can be further simplified:

\[ C^{a'a'}(\tau) = \left\langle F^a_0(\tau)F^a_0(0) \right\rangle \equiv C^{a'a'}(\tau) \]  
\[ (2.35) \]

Since \( F_0 \) is proportional to the spherical harmonic \( Y_{20} \), \( F_0 \) can be expressed in a molecule-fixed coordinate system \( (F_{0}^{\text{int}}) \) in combination with a time-dependent rotation relating the molecule-fixed to the laboratory frame. Use of the Wigner rotation element \( D^{2}_{0l}(\Theta^a_{\text{mol}}) \) gives:

\[ C^{a'a'}(\tau) = \sum_{l,l'=0}^{2} \left\langle D^{2}_{0l}(\Theta^a_{\text{mol}}(\tau))D^{2*}_{0l'}(\Theta^a_{\text{mol}}(0))F^a_{l'}^{\text{int}}(\tau)F^a_{l}^{\text{int}}(0) \right\rangle \]  
\[ (2.36) \]

If the overall molecular tumbling is independent from the internal motions, equation 2.36 can be written as

\[ C^{a'a'}(\tau) = \sum_{l,l'=0}^{2} \left\langle D^{2}_{0l}(\Theta^a_{\text{mol}}(\tau))D^{2*}_{0l'}(\Theta^a_{\text{mol}}(0))F^a_{l'}^{\text{int}}(\tau)F^a_{l}^{\text{int}}(0) \right\rangle \]  
\[ (2.37) \]

It must be stressed that macromolecular dynamics are generally very complex. In particular, in partially folded or unfolded molecules, mode coupling and/or local diffusion must be accounted for. The formalism presented in the following is sufficient for quantitative NOE investigation in globular proteins. \( \left\langle D^{2}_{0l}(\Theta^a_{\text{mol}}(\tau))D^{2*}_{0l'}(\Theta^a_{\text{mol}}(0)) \right\rangle \) may be expressed in terms of the overall correlation time \( 1/\tau_c \) under the assumption of isotropic molecular diffusion (which is a very good approximation in practice). Equation 2.37 becomes
\[ C^{aa'}(\tau) = \frac{1}{5} e^{-\tau/\tau_c} \left( \frac{r_{a\text{rigid}}}{r_{a'}} \right)^3 \sum_{i,j=2}^2 4\pi \left( \frac{1}{r_{a'}^{3/2} r_{a}^{3/2}} Y_{3/2,3/2}(\theta_{a'}) Y_{3/2,3/2}^{*}(\theta_a) \right) \]

(2.38)

It has been shown that independence between the fluctuations of the vector length and orientation is a good approximation for many proton-proton NOEs [2,5]. In that case, equation 2.38 simplifies to:

\[ C^{aa'}(\tau) = \left( \frac{r_{a\text{rigid}}}{r_{a'}} \right)^3 \left( \frac{1}{r_{a'}^{3/2} r_{a}^{3/2}} \right) \left( \frac{1}{2} (3\cos^2 \theta_{a,a'} - 1) \right) e^{-\tau/\tau_c} = C^{aa'}_{\text{int}}(\tau)e^{-\tau/\tau_c} \]

(2.39.1)

with

\[ C^{aa'}_{\text{int}} = \left( \frac{r_{a\text{rigid}}}{r_{a'}} \right)^3 \left( \frac{1}{r_{a'}^{3/2} r_{a}^{3/2}} \right) \left( \frac{1}{2} (3\cos^2 \theta_{a,a'} - 1) \right) \]

(2.39.2)

\( \theta_{a,a'} \) is the projection angle between the vectors \( a \) and \( a' \).

2.2.5.1. Simplifications for methyl groups

The general expressions for methyl groups are complex, but they simplify under certain assumptions. For auto-correlated relaxation between two distinct groups of equivalent spins the spectral density function is (see equation 2.29)

\[ J_{\jmath}^D(\omega) = \tau_{\text{max}} \int_0^{\tau_{\text{max}}} C^{\text{D}D}_{\jmath} (\tau) \cos(\omega \tau) d\tau \]

(2.40)

A similar simplification as used in equation 2.39 is not possible. If the molecular tumbling is isotropic, the correlation function is
If one group consists of a single spin

$$C^{\text{D}(\bar{y})}(\tau) = e^{-\tau \tau_{c}} (r_{y}^{\text{rigid}})^{6} \sum_{l,l'=2}^{2} \frac{1}{N_{i}N_{j}} \sum_{x=1}^{N_{i}} \sum_{y=1}^{N_{j}} \left< \frac{Y_{2l'}(\theta_{i_{x},i_{y}}(\tau))Y_{2l}(\theta_{i_{x},i_{y}}(0))}{r_{i_{x},i_{y}}^{3}(\tau)r_{i_{x},i_{y}}^{3}(0)} \right>$$ \hspace{1cm} (2.41)$$

In more sophisticated models one internal correlation time is assigned to the rotation around $C_{3}$ and a second one to the fluctuation of $C_{3}$, which are then used in the extended Lipari-Szabo formalism [2.6], or a three-site jump-model with defined jump rates is employed. The jump model (vide supra) can be implemented with a $N \times N$ submatrix for an $N$-site exchange. The magnetization vector is enlarged by two dimensions per methyl group.

2.2.6. Lipari-Szabo approximation

Lipari and Szabo proposed to approximate the correlation function of the internal motion with a monoexponential decay with a single internal correlation time $\tau_{\text{int}}$ [2.7]. At infinite time, the correlation function reaches the Lipari-Szabo order parameter. Practically, this order parameter is only sensitive to fast motion ($\tau_{\text{int}} << \tau_{c}$). Since in experiment the spectral density function integrates $C_{\text{int}}^{\text{var}}(\tau)$ over the NOESY mixing time $\tau_{\text{mix}}$ rather than the commonly assumed infinity, this order parameter for fast motion actually is $C_{\text{int}}^{\text{var}}(\tau_{\text{mix}})$. This distinction bears an important implication for the dependence on slow motion (vide infra). For practical purposes, the order parameter of fast motion is here defined as:
\[
\left( S_{aa', \text{fast}}^{\text{fast}, \text{fast}} \right)^2 \equiv \left\langle C_{\text{int}}^{aa'}(\tau_{\text{mix}}) \right\rangle \tag{2.43}
\]

The correlation function becomes
\[
C_{aa'}^{\text{fast}}(\tau) = e^{-\tau/\tau_c} \left[ \left( S_{aa', \text{fast}}^{\text{fast}, \text{fast}} \right)^2 + \left\langle C_{\text{int}}^{aa'} \right\rangle - \left\langle S_{aa', \text{fast}}^{\text{slow}, \text{fast}} \right\rangle^2 \right] e^{-\tau/\tau_{\text{int}}} \tag{2.44}
\]

\( C_{aa'}^{\text{fast}}(\tau) \) still contains an ensemble average which extends over all molecules. If there is additional slow motion present (\( \tau_{\text{int}} \gg \tau_c \)), not all conformations are sampled by a single molecule during \( \tau_c \). If \( \tau_{\text{mix}} > \tau_{\text{int}} \), this requires that the averaging of \( C_{\text{int}}^{aa'}(\tau) \) over all molecules has to be maintained.

If the motion is even slower, that is \( \tau_{\text{mix}} < \tau_{\text{int}} \), the ergodic hypothesis breaks down. The averaging happens at the level of spectral intensities instead of the relaxation rates. In practice, it is usually reasonable to assume that the fast order parameter of a specific molecule is equal to the average over all molecules and the brackets can be omitted in equation 2.43.

Since dipolar auto-correlated relaxation is the dominant mechanism in NOESY spectroscopy, only the case of \( a = a' = D(i,j) \) is discussed further. Insertion of expression 2.44 into equation 2.33 yields for the spectral density function
\[
J_{D(i,j), \text{fast}}^{\text{fast}}(\omega) = \left( S_{D(i,j), \text{fast}}^{\text{fast}, \text{fast}} \right)^2 \frac{\tau_c}{1 + (\tau_c \omega)^2} + \left( \frac{1}{\tau_{ij}^{\text{rigid}}} \right)^6 \left( S_{D(i,j), \text{fast}}^{\text{fast}, \text{fast}} \right)^2 \frac{\tau_{\text{eff}}}{1 + (\tau_{\text{eff}} \omega)^2} \tag{2.45}
\]

with
\[
\frac{1}{\tau_{\text{eff}}} = \frac{1}{\tau_c} + \frac{1}{\tau_{\text{int}}} \tag{2.46}
\]

\( \left( S_{D(i,j), \text{fast}}^{\text{fast}, \text{fast}} \right)^2 \) is the order parameter for fast internal motion of the dipolar vector:


\[
(S^{D(i),\text{fast}})^2 \equiv \left( r_{ij}^{\text{rigid}} \right)^6 \frac{4\pi}{5} \sum_{q=-2}^{2} \left( \frac{Y_{2q} (\theta_{ij}, \phi_{ij})}{(r_{ij}^{(\text{mix})})^3} \right) \left( \frac{Y_{2q}^* (\theta_{ij}, \phi_{ij})}{(r_{ij}^{(0)})^3} \right)
\]

\[
\approx \left( r_{ij}^{\text{rigid}} \right)^6 \frac{4\pi}{5} \sum_{q=-2}^{2} \left( \frac{Y_{2q} (\theta_{ij}, \phi_{ij})}{(r_{ij}^{(\text{mix})})^3} \right) \left( \frac{Y_{2q}^* (\theta_{ij}, \phi_{ij})}{(r_{ij}^{(0)})^3} \right)^2
\]

(2.47)

2.2.7. Practical expressions

The simplest and most common way to extract distances from the measured cross-relaxation rate constant is to use equations 2.25.3 and 2.29 under the assumption of a rigid molecule and exclusive retention of the spectral density function sampling the frequency \( |\omega_i - \omega_j| \approx 0 \) (valid for \( 1 \ll (\tau_c \omega)^2 \)). Motional effects are absorbed into the distance, which must be replaced by an effective distance \( r_{ij}^{\text{eff}} \). In the following, NOEs involving single spins and groups of equivalent spins are treated separately.

2.2.7.1. NOEs and distances between single spins

From equation 2.25.3 follows

\[
\sigma_{ij} = -\left( \frac{\mu_B}{4\pi} \right)^2 \frac{\gamma^4 h^2}{10} \frac{\tau_c}{(r_{ij}^{\text{eff}})^6}
\]

(2.48)
More commonly, the distance is extracted from the cross-relaxation rate constant. The effective distance is then

$$r_{ij}^{\text{eff}} = \left( -\frac{\mu_0}{4\pi} \frac{\gamma^4 \hbar^2}{10 \sigma_y} \tau_c \right)^{1/6} = \left( -56.94 \frac{\tau_c}{\text{ns}} \right)^{1/6} \text{Å}$$

(2.49)

If the molecular correlation time and the measured cross-relaxation rate constant are inserted in units of nanoseconds and of inverted seconds, respectively, the distance is obtained in units of Ångstrom. The relationship between the distances in a rigid molecule and the effective distances can be expressed with the order parameter:

$$r_{ij}^{\text{eff}} = \frac{r_{ij}^{\text{rigid}}}{\left(S^{\text{D}(i,j),\exp}\right)^{1/3}}$$

(2.50)

Note that this holds true for motions on all timescales.

It is noted that an exact extraction of the effective distance requires an accurate measurement of $\tau_c$. It is recommended to determine exact values of $\tau_c$ with $^{15}$N longitudinal and transverse relaxation measurements.

All studies employ one of three approaches to use the effective experimental distance in structure calculations. The first and foremost way is to ignore motional effects completely and relegate their impact into the experimental error and concomitantly to set large upper (and rarely also lower) limits:

$$\left(S^{\text{D}(i,j),\exp}\right)^2 = 1$$

(2.51.1)

and

$$r_{ij}^{\text{eff}} = r_{ij}^{\text{rigid}}$$

(2.52.1)
Despite of its simplicity, for the majority of NOEs this approximation is good.

Secondly, the simplest model accounting for motional effects is the so-called $<r^6>$ averaging. All motion is treated as if it were slow and equation 2.49 gives the order parameter.

$$\left( S^{D(\bar{\bar{J}}),exp} \right)^2 = \left( r_{ij}^{rigid} \right)^6 \left\langle \frac{1}{r_{ij}^6} \right\rangle$$

(2.51.2)

and

$$r_{ij}^{\text{eff}} = \left\langle \frac{1}{r_{ij}^6} \right\rangle^{-1/6}$$

(2.52.2)

It is clear that the contribution to $r^{\text{eff}}$ of temporarily short distances to the NOE is overestimated if part of the motion is in fact fast. In an ensemble structure calculation, true short distances are rejected such that the structure becomes ‘deflated’.

In a third approach, $<r^3>$ averaging is employed. All motion is treated as if it were fast, but the angular dependence is ignored.

$$\left( S^{D(\bar{\bar{J}}),exp} \right)^2 = \left( r_{ij}^{rigid} \right)^3 \left\langle \frac{1}{r_{ij}^3} \right\rangle^2$$

(2.51.3)

and

$$r_{ij}^{\text{eff}} = \left\langle \frac{1}{r_{ij}^3} \right\rangle^{-1/3}$$

(2.52.3)

On one hand, this model overestimates contributions to the NOE because it neglects the angular dependence of the fast motion. On the other hand, it underestimates contributions from slow
motion. It is not easy to quantify the overall effect on the structure calculation. Note that the model is a compromise such that a priori structural knowledge is not required.

2.2.7.2. NOEs and distances between groups of equivalent spins

Although most current structure calculation software packages use simple $<r^6>$ averaging (or summation) for interpretation of NOEs involving groups of equivalent spins (vide infra), here the pseudo-atom model will be discussed first. From equation 2.29 follows

$$\sigma_{ij} = -\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma^4 h^2}{10} \frac{N_i \tau_s}{(r_{eff}^i)^6}$$

(2.53)

and the effective distance between the pseudoatoms becomes

$$r_{eff}^i = \left(-56.94 N_i \frac{\tau_s}{\sigma_{ij}} \frac{\text{ns}}{\text{s}^{-1}}\right)^{1/6} \text{Å}$$

(2.54)

Even if the multiplicity effect of the spins is absorbed into the effective distance between the pseudoatoms, a distinction is made between the dynamics within the groups of equivalent spins and between them. The effect of intra-group dynamics can be treated with equations 2.41 and 2.42. This averaging is employed in all three cases outlined above (rigid model, $<r^6>$ averaging and $<r^3>$ averaging). Once this averaging is carried out, the cases described by equations 2.50-52 are applied. The type of the intra-group averaging depends on the spin group and is in principle independent of the type of model given by 2.50-52. The procedure is obviously somewhat cumbersome. It is therefore convenient to calculate maximum errors induced by the intra-group motion if the pseudoatom is employed in a structure calculation. Then, equations 2.50-2.52 are
also valid for groups of equivalent spins, while the maximum error is absorbed into the allowed upper and lower limits for the structure calculation.

The exact impact of intra group motion is difficult to predict because it depends on exact geometries. For example, an NOE to and from a methyl group depends not only on the distance but also on the orientation of the C$_3$ axis. Thus, an \textit{a priori} known structure is needed. Several authors calculated maximum allowed distances between pseudo-atom positions and individual group atoms in an amino-acid specific manner. In methyl and methylene groups the pseudoatom is placed centrally with respect to the protons. The pseudoatom representing both methyl groups in valine and leucine is located in the center of all six protons. These limits can be narrowed if it is assumed that the protons exchange on a fast timescale. A summary of the maximum possible motion-corrected deviations of the positions of the pseudoatoms from the individual atoms, $\bar{x}_Q$ and $\bar{x}_\kappa$, is provided in Table 1.

It is noteworthy that it has been shown that direct structural refinement over the distances between all individual atoms in a group puts narrower restraints on these groups and avoids the need for using pseudoatoms. This approach is only feasible with $\langle \bar{r}^6 \rangle$ averaging (or $\langle r^6 \rangle$ summation); fast motion may be treated incorrectly, but, as is also the case when using the pseudoatom approach, the result is that affected constraints are set slightly more loosely than could be done if a fully detailed treatment of such motions were available.

\textbf{Table 1.} Pseudoatom corrections for experimentally determined distances between a single atom and a group of equivalent atoms.

<table>
<thead>
<tr>
<th>correction$^a$ [Å]</th>
<th>CH$_3$</th>
<th>CH$_2$</th>
<th>CH$_2$-CH$_3$</th>
<th>aromatic centre</th>
</tr>
</thead>
<tbody>
<tr>
<td>-0.20; 0.40</td>
<td>-0.20; 0.70</td>
<td>1.50</td>
<td>2.48</td>
<td></td>
</tr>
</tbody>
</table>

$^a$For distances between two pseudo-atoms it is recommended to sum the individual corrections.

$^b$If two values are separated by a semicolon, the first and the second value are the corrections to the lower and upper limit, respectively.
2.2.7.3. Expressions for molecular dynamics simulations

It is common to compare experimental results with molecular dynamics simulations. Sometimes, NOEs are also used to generate restraints $r_{ij}^{\text{exp}}$ for MD simulations by including an NOE term in the force field, $V_{ij}^{\text{NOE}}$, as (in its simplest form)

$$V_{ij}^{\text{NOE}}(r_{ij}^{\text{MD}}) = \frac{1}{2} K^{\text{NOE}} (r_{ij}^{\text{MD}} - r_{ij}^{\text{exp}})^2 \quad \text{if } r_{ij}^{\text{MD}} > r_{ij}^{\text{exp}}$$

$$V_{ij}^{\text{NOE}}(r_{ij}^{\text{MD}}) = 0 \quad \text{if } r_{ij}^{\text{MD}} \leq r_{ij}^{\text{exp}}$$

(2.55)

with the force constant $K^{\text{NOE}}$. More sophisticated forms may have linear dependencies for large violations or avoid forth-power terms, or the back-calculated values are forced not to be in worse agreement than the previous best ones, so that they eventually approach the experimental values.

Since the trajectories contain time-ordered snapshots of the structure the correct prediction of NOEs from MD must be made with correct averaging. The simplest averaging procedure is the discrete $<r^6>$ averaging:

$$r_{ij}^{\text{MD}} = \left( \frac{1}{N} \sum_{a=1}^{N} \frac{1}{r_{ij,a}^6} \right)^{-1/6}$$

(2.56)

We have also employed $<r^3>$ averaging including the angular dependence

$$r_{ij}^{\text{MD}} = \left( \frac{1}{N} \sum_{a=1}^{N} \frac{1}{r_{ij,a}^3} \sum_{b=1}^{N} P_2 \left( \cos \theta_{\text{int,ab}}^M \right) \frac{1}{r_{ij,b}^3} \right)^{-1/6}$$

(2.57)

where $a$ and $b$ run over the $N$ structures in the MD set, $P_2$ is the Legendre polynomial of second order and $\theta_{\text{int,ab}}^M$ denotes the projection angle between the two internuclear vectors.

It has been proposed to use a memory function with a characteristic decay time $\tau_{\text{mem}}$. 
\[ r_{ij}^{\text{MD}}(t) = \left( \frac{1}{\tau_{\text{mem}}} \int_{0}^{t} e^{-t'/\tau_{\text{mem}}} \left[ r_{ij} \left( t-t' \right) \right]^{q} dt' \right)^{1/q} \] (2.58)

where \( q \) is either 3 or 6. Not surprisingly, structure refinements on test data sets have shown that time-averaged methods lead to more truthful pictures of conformational sampling. As typical MD trajectories are no longer than a few nanoseconds, a single copy of the molecule is likely not to sample the complete conformational space. In that case, the averaging can be carried out over multiple copies of the molecule. Combining equations 2.56-58 with ensemble averaging gives:

\[ r_{ij}^{\text{MD(time,space)}}(t) = \left( \frac{1}{\tau_{\text{mem}}} \int_{0}^{t} e^{-t'/\tau_{\text{mem}}} \left[ \langle r_{ij} \left( t-t' \right) \rangle \right]^{q} dt' \right)^{1/q} \] (2.59)

The ensemble averaging is Boltzmann-weighted, ideally using the free energy. In practice, it is easier to use the potential energy also averaged with a short memory function.

It is often more convenient to write the correlation function as an expression of Cartesian coordinates:

\[ C(t) = \frac{1}{6} \begin{bmatrix} 3x^2(0) - r^2(t) & 3y^2(0) - r^2(t) & 3z^2(0) - r^2(t) \\ 3x^2(t) - r^2(t) & 3y^2(t) - r^2(t) & 3z^2(t) - r^2(t) \\ x y(0)x(t) + 9x y(0)x(t) + 9y z(0)y(t) + 9y z(0)y(t) \end{bmatrix} \] (2.60)
3. Pulse sequences

In this chapter, we first introduce the basic NOESY building block, which is the two-dimensional $[^1\text{H},^1\text{H}]-\text{NOESY}$. In Chapter 3.1, we analyse the magnetization flow using the product operator formalism. As outlined in the previous chapter, the NOE transfer is a relaxation phenomenon and is therefore only expressed by transfer functions. The addition of a $^{13}\text{C}$ and/or $^{15}\text{N}$ dimension is treated in Chapter 3.2 with the focus on practical aspects.

3.1. The basic 2D NOESY pulse sequence

![Basic pulse sequence scheme of the 2D $[^1\text{H},^1\text{H}]-\text{NOESY}$ experiment. The black bars indicate non-selective 90° pulses. A typical phase cycle is: $\phi_1 = \{x, x, x, -x, -x, -x, -x\}; \phi_2 = \{x\}; \phi_3 = \{x, y, -x, -y\}; \phi_{\text{rec}} = \{x, y, -x, -y, -x, -y, x, y\}$. Note that this cycle suppresses axial peaks. Quadrature detection in the $t_1$ dimension is achieved by the States-TPPI method applied to the phases $\phi_i$ and $\phi_{\text{rec}}$.](image)

In this section, the mathematical description of the basic 2D NOESY pulse sequence is given (Figure 3.1). It as assumed that a two spin-1/2 system $ij$ is not scalar coupled. Equilibrium magnetization $I_{i,z} + I_{j,z}$ is established prior to the first pulse. The first pulse brings the magnetization into the transverse plane:
\[ I_{i,z} + I_{j,z} \rightarrow -I_{i,y} - I_{j,y} \] (3.1)

During \( t_1 \), evolution under the chemical shifts results in

\[ -I_{i,y} - I_{j,y} \rightarrow -\cos(\omega t_1)I_{i,y} - \cos(\omega t_1)I_{j,y} \] (3.2)

The second pulse brings the magnetization that is parallel to the y-axis back along the z-axis (the magnetization parallel to the x-axis is not detected in the final spectrum and is neglected).

\[ -\cos(\omega t_1)I_{i,y} - \cos(\omega t_1)I_{j,y} \rightarrow -\cos(\omega t_1)I_{i,z} - \cos(\omega t_1)I_{j,z} \] (3.3)

The fate during the mixing period \( \tau_{\text{mix}} \) is governed by the Solomon equations as indicated by the transfer function \( T_{ij}^{\text{NOE}} \), which will be explicitly given in Chapter 4. \( T_{ij}^{\text{NOE}} \) gives raise to cross peaks for \( i \neq j \) and to diagonal peaks for \( i = j \), respectively.

\[ -\cos(\omega t_1)I_{i,z} - \cos(\omega t_1)I_{j,z} \]
\[ \rightarrow -\cos(\omega t_1)[T_{ii}^{\text{NOE}}(\tau_{\text{mix}})I_{i,z} + T_{ij}^{\text{NOE}}(\tau_{\text{mix}})I_{j,z}] - \cos(\omega t_1)[T_{jj}^{\text{NOE}}(\tau_{\text{mix}})I_{j,z} + T_{ji}^{\text{NOE}}(\tau_{\text{mix}})I_{i,z}] \] (3.4)

The third pulse brings the magnetization back into the transverse plane, where it evolves during the time \( t_2 \) and is detected in quadrature:

\[ -\cos(\omega t_1)[T_{ii}^{\text{NOE}}(\tau_{\text{mix}})I_{i,z} + T_{ij}^{\text{NOE}}(\tau_{\text{mix}})I_{j,z}] - \cos(\omega t_1)[T_{jj}^{\text{NOE}}(\tau_{\text{mix}})I_{j,z} + T_{ji}^{\text{NOE}}(\tau_{\text{mix}})I_{i,z}] \]
\[ \rightarrow -\cos(\omega t_1)[\exp(i\omega t_2)T_{ii}^{\text{NOE}}(\tau_{\text{mix}})I_{i,y} + \exp(i\omega t_2)T_{ij}^{\text{NOE}}(\tau_{\text{mix}})I_{j,y}] \]
\[ -\cos(\omega t_1)[\exp(i\omega t_2)T_{jj}^{\text{NOE}}(\tau_{\text{mix}})I_{j,y} + \exp(i\omega t_2)T_{ji}^{\text{NOE}}(\tau_{\text{mix}})I_{i,y}] \] (3.5)
If the first pulse is shifted by 90°, an analogous expression is obtained that is modified by a sine term during $t_1$. The two scans can be combined to yield a complex frequency modulation in $t_1$ and $t_2$:

$$
-\exp(i\omega_i t_1)[\exp(i\omega_j t_2)T_{ij}^{\text{NOE}}(\tau_{\text{mix}})I_{i,j} + \exp(i\omega_j t_2)T_{ji}^{\text{NOE}}(\tau_{\text{mix}})I_{j,i}]
-\exp(i\omega_j t_1)[\exp(i\omega_i t_2)T_{ji}^{\text{NOE}}(\tau_{\text{mix}})I_{j,i} + \exp(i\omega_i t_2)T_{ji}^{\text{NOE}}(\tau_{\text{mix}})I_{i,j}]$$

(3.6)

The four terms give rise to four NOESY peaks. Fourier transformation produces two-dimensional delta functions centred at the frequencies $(\omega_i, \omega_i)$, $(\omega_i, \omega_j)$, $(\omega_j, \omega_i)$, $(\omega_j, \omega_j)$ with relative intensities $T_{ii}^{\text{NOE}}(\tau_{\text{mix}})$, $T_{ij}^{\text{NOE}}(\tau_{\text{mix}})$, $T_{ji}^{\text{NOE}}(\tau_{\text{mix}})$, and $T_{jj}^{\text{NOE}}(\tau_{\text{mix}})$. Obviously, the first and third peaks are the diagonal peaks of spins $i$ and $j$, while the second and fourth are the cross peaks connecting $i$ and $j$. Note that the peaks would have finite linewidths if relaxation during the chemical shift evolution periods was included. The first 2D NOESY ever recorded is shown in Figure 3.2.
3.2. Heteronucleus-resolved NOESY

The introduction of isotope-labelling of heavy atoms enabled development of a large arsenal of heteronuclear three- and higher-dimensional NOESY experiments that significantly simplify the analysis of proton-proton NOEs and increase the number of distance restraints. As of now,
series of 3D $^{15}$N-resolved or 3D $^{15}$N,$^{13}$C-resolved $^{1}$H,$^{1}$H]-NOESY spectra have been used in our laboratory to extract exact NOEs (eNOEs) (see Figure 3.3). Two main types of these experiments, the NOESY-HXQC and HXQC-NOESY schemes, are distinguished, where X stands for S (single) or M (multiple). In NOESY-HXQC, $^{15}$N,$^{1}$H]-HXQC and $^{13}$C,$^{1}$H]-HXQC elements are employed simultaneously after proton chemical shift evolution and $^{1}$H,$^{1}$H]-NOE mixing (Figure 3.3a). In HXQC-NOESY, parallel $^{15}$N,$^{1}$H]-HXQC and $^{13}$C,$^{1}$H]-HXQC elements including heteronuclear and proton chemical shift evolutions are employed before $^{1}$H,$^{1}$H]-NOE mixing (Figure 3.3b). The choice of the order of the elements gains an additional significance for the measurement of eNOEs. The intensity of detected $^{1}$H magnetization $I_{ij}$ originating from the initial $^{1}$H$_i$ magnetization with intensity $I_{init}$ can be expressed as follows for HXQC-NOESY and for NOESY-HXQC:

\[ I_{ij} \left( \tau_{mix} \right) = \alpha_{ij}^{\text{rec}} I_{init}^{\text{NOE}} T_{ij}^{\text{HXQC}} T_{ij}^{\text{NOE}} \left( \tau_{mix} \right) T_{ij}^{\text{WS}} \]  

(3.7)

\[ I_{ij} \left( \tau_{mix} \right) = \alpha_{ij}^{\text{rec}} I_{init}^{\text{NOE}} T_{ij}^{\text{HXQC}} \]  

(3.8)

$\alpha_{ij}^{\text{rec}}$ is the fractional part of the magnetization that has recovered during the interscan delay, $T_{ij}^{\text{HXQC}}$ the loss of magnetization during the HXQC element, and $T_{ij}^{\text{WS}}$ the loss of magnetization during the water suppression element.

To assess experimentally the effects of swapping the HXQC and NOESY elements both pulse sequences were run and analysed for a triple-labelled GB3 sample. Virtually no systematic error is introduced by swapping. If the cross-relaxation rate constants in both transfer directions are determined individually for the NOESY-HSQC experiment, the rmsd between the two symmetry-related cross-relaxation rate constants is circa twice as large as in the HMQC-NOESY experiment (0.82 s$^{-1}$ versus 0.35 s$^{-1}$). However, if both cross peaks can be evaluated, equation
3.10 can be used (vide infra) and the obtained NOE rates are equally accurate as those from the HXQC-NOESY experiment.

**Figure 3.3.** Pulse sequences of the 3D \([^{15}\text{N},^{13}\text{C}]-\text{resolved} \quad [^{1}\text{H},^{1}\text{H}]-\text{NOESY} \) experiments for the measurement of NOE build-ups. Narrow and wide black bars indicate non-selective 90° and 180° pulses. The white bar and the curved shape indicate a trim pulse of 1 ms duration and a Gaussian-shaped 90° pulse of 1 ms duration, respectively. The delay $\tau = 1.7$ ms is optimized for $^{1}\text{H} - ^{13}\text{C}$ transfers. Unless indicated otherwise, all radio-frequency pulses are applied.
with phase x. a) 3D $[^{15}\text{N},^{13}\text{C}]$-resolved NOESY-HSQC experiment. Alternatively, $\tau$ can be set to 2.7 ms and the $^{13}\text{C}$ 180° pulses during the transfers are shifted by 1 ms to optimize both the $^1\text{H}-^{15}\text{N}$ and $^1\text{H}-^{13}\text{C}$ transfers. The phase cycle is: $\phi_1 = \{x, x, x, x, -x, -x, -x, -x\}; \phi_2 = \{x, x, -x, -x\}; \phi_3 = \{x, -x\}; \phi_4 = \{x, -x\}; \phi_{\text{rec}} = \{-x, x, x, -x, x, x, -x, x\}$. b) 3D $[^{15}\text{N},^{13}\text{C}]$-resolved HMQC-NOESY experiment. If only $^{15}\text{N}$ resolution is desired, the $^{13}\text{C}$ sequence is replaced by the one indicated in the box and $\tau = 2.7$ ms. The phase cycle is: $\phi_1 = \{x, x, -x, -x\}; \phi_3 = \{x, -x\}; \phi_4 = \{x, -x\}; \phi_{\text{rec}} = \{-x, x, x, -x\}$. In all experiments, quadrature detection in the $^1\text{H}(t_1)$ and simultaneous $^{15}\text{N}/^{13}\text{C}(t_2)$ dimensions is achieved by the States-TPPI method applied to the phases $\phi_1, \phi_3, \phi_4$ and $\phi_{\text{rec}}$.

3.2. 1. Practical recommendations for eNOE measurements

Typical experiments require four to six mixing times $\tau_{\text{mix}}$ with a maximum value of ca. $5 \times 10^{-10}$ s$^2/\tau_c$ for deuterated samples and $2.5 \times 10^{-10}$ s$^2/\tau_c$ for protonated samples. The spectra are recorded with $200(^1\text{H}^{\text{indirect}}) \times 40(^{15}\text{N}/^{13}\text{C}^{\text{indirect}}) \times 1024(^1\text{H}^{\text{direct}})$ complex points, maximum evolution times $t_{\text{max}, ^1\text{H}} = 22$ ms, $t_{\text{max}, ^{15}\text{N}} = 14$ ms, $t_{\text{max}, ^{13}\text{C}} = 8$ ms, and $t_{\text{max}, ^1\text{H}} = 102$ ms, an interscan delay of 1 s and 4 scans per increment, which results in a measurement time of 1.5 days per spectrum (the full 8-step phase cycle may be too long). Of course, several parameters must be adjusted to the specific case, such as a longer interscan delay for larger proteins, etc. $^{15}\text{N}$ decoupling may be achieved with GARP and $^{13}\text{C}$ decoupling with a p5m4 supercycle, using CHIRP pulses for example.

The measurement of eNOEs is very sensitive to the slightest distortion of the baseline. As each spectrum is unique in terms of water suppression, number of points, etc., we do not have a general recipe. Typically, we use routines that automatically choose the appropriate order of a polynomial correction function that is applied in the proton dimensions, and no correction is applied in the $^{15}\text{N}/^{13}\text{C}$ dimension. If the water signal causes severe distortions in the direct
dimension, additional correction functions may be applied independently on either side of the water signal.

3.3. Normalization to diagonal peak intensities

If the NOESY element is placed after the HXQC element, relaxation during HXQC is identical for the diagonal and every of its cross peaks since they share the same magnetization pathway (as long as $T_{ij}^{WS}$ can be assumed identical). $T_{ij}^{NOE}$ can now be extracted by normalization of the cross-peak intensity ($i \neq j$) to the diagonal peak intensity ($i = j$) at $t = 0$:

$$T_{ij}^{NOE}(\tau_{mix}) = \frac{I_{ij}(\tau_{mix})}{I_{ii}(0)}$$  \hspace{1cm} (3.9)

The advantage of this approach is that $\sigma_{ij}$ and $\sigma_{ji}$ are obtained independently and can be used for error estimation. In addition, if one of the pathways cannot be evaluated (e.g. due to peak overlap) the cross-relaxation rate constant can still be obtained from the other. On the other hand, if quantitative NOEs of aliphatic protons are evaluated, the water suppression sequence (typically WATERGATE or a binomial suppression) suppresses in part the $^1$H$^\alpha$ signals, which results in frequency-dependent $T_{ij}^{WS}$ values and hence the assumption of uniformity is no longer valid. Similarly, if signal intensities rather than volumes are analysed the non-uniform linewidth of various proton types also alters $I_{ij}$. If one of the cross peaks is missing no value can be obtained or a larger error has to be tolerated. In our experience, superior water suppression is achieved with NOESY-HSQC experiments, where gradients and trim pulses can be used. In cases where both
cross peaks can be analysed, more exact values of cross-relaxation rate constants obtained from HXQC-NOESY with non-uniform $T_{j}^{WS}$ and all NOESY-HXQC can be calculated from

$$T_{j}^{\text{NOE}}(\tau_{\text{mix}}) = T_{ji}^{\text{NOE}}(\tau_{\text{mix}}) = \frac{I_{ji}(\tau_{\text{mix}})I_{ji}(\tau_{\text{mix}})}{I_{ii}(0)I_{jj}(0)}$$

(3.10)

Another way to deal with the different magnetization pathways in NOESY-HXQC experiments is to determine $T_{ij}^{\text{HXQC}}$ from a 2D HXQC experiment or to simulate it based on predicted relaxation rates. For a conventional HSQC element, $T_{ij}^{\text{HXQC}}$ is mostly determined by the transverse relaxation rates of $^{1}H_{j}$ and $^{15}N_{j}$ or $^{13}C_{j}$.

3.4. Correction for deuteration

Once the cross-relaxation rate constant is obtained by fitting, it has to be corrected for deuteration effects if exchangeable atoms (mostly amide protons) are in exchange with solvent deuterons (see Figure 3.4). The most common solvent is a D$_2$O/H$_2$O mixture since deuterated water is added for the lock signal. Very importantly, the correction is only applied if the spin that receives the magnetization is exchangeable (if the spin from which the initial magnetization originates is exchangeable, $I(0)$ is also reduced and the effect is already accounted for in equations 3.9 and 3.10). The true cross-relaxation rate constant is then obtained as:

$$\sigma_{ij}^{\text{true}} = \frac{\sigma_{ij}^{\text{fit}}}{1 - \text{Deut} / 100\%}$$

(3.11)
*Deut* is the D$_2$O level in per cent (%). In our experience, 3 % D$_2$O is sufficient for the lock signal and causes minimal signal losses in NOESY.

---

**Figure 3.4.** The effect of partial deuteration of the solvent on apparent cross-relaxation rates. The situation where the initial magnetization is on an exchangeable proton is shown in **a)**, and where the receiving proton is exchangeable in **b)**. If the solvent is partially deuterated and exchanges with the molecule under study, the apparent NOE may be described by a superposition of two microscopic possibilities. The diagonal and cross peaks expected in a 2D NOESY spectrum after 0 and finite mixing times are schematically depicted on the left- and right-hand side, respectively. It is seen that in case **a)** both peaks are scaled by (1-Deut), whereas in case **b)** only the cross peak is rescaled and a correction has to be applied to the apparent cross-relaxation rate.
4. Extraction of exact NOE rates and distances

In this chapter, two procedures to extract exact NOE rate constants are discussed. In practice, it turns out that the most successful approaches depend on iterative protocols, where preliminary values of the NOE rate constants are used to calculate preliminary structures (alternatively, an X-ray structure may be used). These, in turn, are used to back-predict theoretical NOEs, which are then used to improve the experimentally obtained values.

4.1. Spin diffusion

All approaches to extract exact cross-relaxation rate constants make use of the simplifications inherent to the Solomon equations presented in equation 2.30 (which may be extended as in equation 2.32 if exchange causes appearance of additional peaks). For a system with \(N\) spins 1/2, a 2D NOESY pulse sequence produces in principle up to \(N \times N\) peaks of which \(N\) are diagonal peaks and \(N \times (N-1)\) are cross peaks. The evolution of the matrix \(I(t)\) containing the volumes or intensities of these peaks (not to be confused with magnetization operators) is obtained from equation 2.30.

\[
\frac{d}{dt} I(t) = -RI(t)
\]  

(4.1)

where the intensity matrix is
\[
I(t) = \begin{pmatrix}
I_{11}(t) & I_{12}(t) & \cdots & I_{11}(t) \\
I_{21}(t) & I_{22}(t) & \cdots & \vdots \\
\vdots & \vdots & \ddots & \vdots \\
I_{N1}(t) & \cdots & I_{N1}(t) & I_{NN}(t)
\end{pmatrix}
\]

At \( t = 0 \), all off-diagonal elements are zero and the diagonal elements give the intensities at the outset of the NOESY mixing period. In what follows, \( R \) designates the relaxation matrix of the differential equation in the reduced space spanned by longitudinal single-spin order operators:

\[
R \equiv \begin{pmatrix}
\rho_1 & \sigma_{12} & \cdots & \sigma_{1N} \\
\sigma_{12} & \rho_2 & \cdots & \sigma_{2N} \\
\vdots & \vdots & \ddots & \vdots \\
\sigma_{1N} & \sigma_{2N} & \cdots & \rho_N
\end{pmatrix}
\]

The formal solution is obviously

\[
I(t) = I(0)e^{-Rt}
\]

and \( R \) is obtained from

\[
R = \ln\left[ I(t)(I(0))^{-1} \right] / -t
\]

Many approaches have been proposed to obtain exact cross-relaxation rate constants. The peak intensities are not only modified by direct magnetization transfer, but also by transfer via a third or more spins. This phenomenon is referred to as ‘spin diffusion’. The transfer pathways are
obtained from the second and higher orders of a Taylor expansion of the exponent in equation 4.4.

\[ I(t) = \left[ 1 - R_t + \frac{1}{2} R^2 t^2 + \ldots \right] I(0) \]  

(4.6)

The first two terms describe the linear-regime build-up. The third term is:

\[ \left[ \frac{1}{2} R^2 i^2 \right]_{ij} = \frac{t^2}{2} \sum_{k=1}^{N} R_{ik} R_{kj} \]  

(4.7)

The diagonal contribution is

\[ \left[ \frac{1}{2} R^2 i^2 \right]_{ii} = \frac{t^2}{2} \sum_{k=1}^{N} R_{ik} R_{ki} = \frac{t^2}{2} \sum_{k=1}^{N} R_{ik}^2 = \frac{t^2}{2} \left( \rho_i^2 + \sum_{k \neq i}^{N} \sigma_{ik}^2 \right) \]  

(4.8)

The second term denotes magnetization that is transferred to neighbouring spins and immediately brought back to the original spin. Contributions to cross peaks are obtained if \( i \neq j \):

\[ \left[ \frac{1}{2} R^2 i^2 \right]_{ij} = \frac{t^2}{2} \sum_{k=1}^{N} R_{ik} R_{kj} = \frac{t^2}{2} \left( \sigma_{ij} (\rho_i + \rho_j) + \sum_{k \neq i, j}^{N} \sigma_{ik} \sigma_{kj} \right) \]  

(4.9)

The first two terms denote magnetization that is transferred directly, with a damping due to autorelaxation of both spins. The third term represents spin diffusion via one additional spin. This contribution to the cross peaks is very important in NOESYs recorded on macromolecules. In particular, the need for a sufficient number of cross peaks often implies that the mixing times must be chosen so that the build-up significantly deviates from the initial linear behaviour. To obtain exact cross-relaxation rate constants, spin diffusion must be accounted for.
Most commonly, a full-matrix approach is used that converts spectral peak volumes or intensities recorded with a single NOESY mixing period into cross-relaxation rate constants by finding the solution of equation 4.4. At first glance, this appears to be the most elegant approach since it accounts correctly for spin diffusion. Indeed, it has been demonstrated in numerous publications that the cross-relaxation rate constants (and the distances) obtained from this approach are more accurate than those obtained from an isolated two-spin approximation (ISPA). There are, however, practical complications. For a macromolecule, the intensities of a majority of the cross peaks are weaker than the spectral noise level. In addition, many peaks are overlapped. In either case, $I$ cannot be fully determined. The only way to correct for this is to supplement $I$ with elements estimated from a preliminary structure obtained from conventional structure calculation methods. Also, it is difficult, if possible at all, to follow the propagation of errors (experimental errors, or those due to lack of data) because the complete system must be treated in one step of calculation. The calculation also requires a symmetric $I$ matrix, which usually requires extensive modifications of the original experimental cross peak intensities. In our experience, it turned out that a non-linear ISPA with subsequent corrections for spin diffusion estimated with a full-matrix approach is superior in practice. On one hand, the data evaluation is straightforward and all NOEs can be analysed in an individual manner. On the other hand, iterative application converges to the solution of the complete full-matrix calculation. An additional advantage is that the approach can easily take partial deuteration of the sample into account. In the following, the original full-matrix approach and the non-linear ISPA with corrections for spin diffusion are discussed. Since the later is novel and used by our laboratory it is discussed in more detail.
4.2. Full relaxation-matrix approach

Because the relaxation matrix $R$ is symmetric (asymmetry due to pseudo atoms is neglected), the evolution of the peak intensities can be calculated by means of matrix diagonalization:

$$I(t) = U_R e^{-D_R t} U_R^{-1} I(0)$$  \hspace{1cm} (4.10)

where the diagonal matrix containing the eigenvalues of the relaxation matrix $R$ is

$$D_R = U_R^{-1} R U_R$$  \hspace{1cm} (4.11)

and $U_R$ contains the eigenvectors. Rather than extracting the rate constants, structural models may then be used to predict intensities (vide infra).

4.2.1. Iterative hybrid relaxation matrix approach with restrained MD/DG

A substantial leap forward constitutes the procedure underlying the IRMA (Iterative Relaxation Matrix Approach) program [4.1,4.2]. At the start, the intensity matrix $I$ is constructed from $R$ which is estimated from a preliminary structure by using equation 4.4. Then, the off-diagonal elements for which the corresponding intensities can be extracted from a NOESY experiment are replaced by them. This hybrid matrix is diagonalized to obtain an updated $R$ matrix using equation 4.5. $R$ is converted into distance constraints and used for a structure refinement by restrained molecular (rMD) simulation (distance geometry (DG) approaches may be used instead). An iteration of these steps is carried out until convergence is achieved. The results for different mixing times are averaged. The strong diagonal peaks are treated purely
theoretically (the leakage contribution is also considered in later versions). More recent versions of the program also consider local mobility such as methyl rotation and ring flips and order parameters of fast motions obtained from MD simulations. A problem is that back-calculation of \( R \) from \( I \) may generate negative eigenvalues. Then, the logarithm in equation 4.5 is inhibited and the eigenvalues may be set to zero or to slightly positive values.

4.3. Non-linear ISPA with correction for spin diffusion

In the first step, all spin pairs giving rise to a cross peak are assumed to form a two-spin system \( i-j \). The exact analytical solution of equation 4.4 is then (diagonalization, exercise 3):

\[
\frac{I_{ij}(t)}{I_{ij}(0)} = \frac{1}{2} \left[ \left( 1 - \frac{\rho_i - \rho_j}{\lambda^+_\text{NOE} - \lambda^-_\text{NOE}} \right) e^{-\lambda^+_{\text{NOE}} t} + \left( 1 + \frac{\rho_i - \rho_j}{\lambda^-_\text{NOE} - \lambda^+_\text{NOE}} \right) e^{-\lambda^-_{\text{NOE}} t} \right] 
\]  

(4.12.1)

\[
\frac{I_{ji}(t)}{I_{ji}(0)} = \frac{1}{2} \left[ \left( 1 + \frac{\rho_i - \rho_j}{\lambda^+_\text{NOE} - \lambda^-_\text{NOE}} \right) e^{-\lambda^+_{\text{NOE}} t} + \left( 1 - \frac{\rho_i - \rho_j}{\lambda^-_\text{NOE} - \lambda^+_\text{NOE}} \right) e^{-\lambda^-_{\text{NOE}} t} \right] 
\]  

(4.12.2)

\[
\frac{I_{ij}(t)}{I_{ij}(0)} \frac{I_{ji}(t)}{I_{ji}(0)} = \frac{-\sigma_{ij}}{\lambda^+_\text{NOE} - \lambda^-_\text{NOE}} \left[ e^{-\lambda^+_{\text{NOE}} t} - e^{-\lambda^-_{\text{NOE}} t} \right] 
\]  

(4.13)

with

\[
\lambda^\pm_{\text{NOE}} = \frac{(\rho_i + \rho_j)}{2} \pm \sqrt{\left( \frac{\rho_i - \rho_j}{2} \right)^2 + \sigma_{ij}^2} 
\]  

(4.14)

For practical purposes, equations 4.12.1 and 4.12.2 are approximated by single-exponential decays (exercise 4):

\[
\frac{I_{ij}(t)}{I_{ij}(0)} = e^{-\rho_{ij} t} 
\]  

(4.15.1)
\[ \frac{I_{ij}(t)}{I_{ij}(0)} = e^{-\rho_j} \]  
\[ (4.15.2) \]

These expressions are independent of the autorelaxation rate of the other spin and of the cross-relaxation rate.

The extraction of the cross-relaxation rates is now carried out in three distinct steps (see Figure 4.1). In the following, the solutions given in equations 4.13 and 4.15 are written as transfer functions \( T^{\text{NOE}} \) introduced in Chapter 3 and may be generalized to groups of magnetically equivalent spins if they occur. First, the autorelaxation rate of spin \( i \), \( \rho_i \), and the intensity at zero mixing time, \( I_{ij}(0) \), is obtained from a two-parameter fit to the diagonal-peak decay. Similarly, \( \rho_j \) and are \( I_{ji}(0) \) obtained.

\[ I_{ii}(t) = T_{ii}^{\text{NOE}}(t)I_{ii}(0) \]  
\[ (4.16.1) \]

\[ I_{jj}(t) = T_{jj}^{\text{NOE}}(t)I_{jj}(0) \]  
\[ (4.16.2) \]

Then, the cross-relaxation rate \( \sigma_{ij} \) between spins \( i \) and \( j \) is obtained by inserting \( I_{ii}(0) \), \( \rho_i \) and \( \rho_j \) into the equation describing the cross-peak build-up with initial magnetization on spin \( i \), and \( I_{jj}(0) \), \( \rho_j \) and \( \rho_i \) into the one describing the cross-peak build-up with the initial magnetization on spin \( j \), respectively:
Figure 4.1. Flow chart outlining the procedure for the determination of eNOE cross-relaxation rates based on the corrections applied to the spectral intensities. As an example, the eNOE originating from the amide H of Gly9 (spin $i$, orange) and enhancing $H^\beta$ of Asn8 (spin $j$, green) of GB3 is shown. (1) The diagonal peak intensities derived from the NOESY spectra are fitted to mono-exponential decay functions to extract the autorelaxation rates $\rho_i$ and $\rho_j$, and the initial intensities on spin $i$, $I_{ii}(0)$. (2) A buildup curve taking into account all magnetization pathways is simulated with the eNORA approach and apparent relaxation rates are determined. The simulation requires previously known 3D structural coordinates as input. Theoretical corrections are applied to the experimental spectral intensities at each mixing time. (3) The NOE buildup is fitted, the quality of the fit is evaluated and distance restraints are generated. A structure calculation may be performed with the new distance restraints. This structure may be used as an input for (2) in a new cycle as indicated by the broken arrow. Note that steps 2 and 3 are reversed if the correction factors are applied to the apparent cross relaxation rate rather than the intensities. Adapted from reference 4.3.
\[ I_{ji}(t) = N_j T_{ji}^{\text{NOE}}(t) I_{ji}(0) \] (4.17.1)

\[ I_{ji}(t) = N_i T_{ji}^{\text{NOE}}(t) I_{ji}(0) \] (4.17.2)

The multiplicities of magnetically equivalent spins \( N_j \) and \( N_i \) must be included if the initial magnetization is transferred to a group of equivalent spins. On the other hand, if the magnetization starts on a group of equivalent spins, the extracted cross-relaxation rate is not affected because the intensities on either side of 4.17.1 and 4.17.2 are scaled accordingly.

The impact of relayed magnetization via neighbour spins on the two-spin solution of cross relaxation can be kept relatively small in practice. It is, however, in general not completely negligible. Thus, in a third step, we calculate the relative contribution from the neighbour spins on the apparent cross-relaxation rate and expressed it as a correction factor \( p \) to the apparent rates. A previously known approximate structure is used and the final cross-relaxation rate is obtained as:

\[ \sigma_{ij} = p_{ij} \sigma_{ij}^{\text{app}} \] (4.18)

Alternatively, a correction factor for each spectral intensity at each mixing time can be calculated and an improved cross-relaxation rate is obtained from a new fit:

\[ I_{ij}(t) = p_{ij}(t) I_{ij}^{\text{app}}(t) \] (4.19)

In this case, steps 2 and 3 are reversed since the cross-relaxation rate is fitted after the calculation of the correction factors.

We have developed two different approaches to determine \( p \) with eNORA (Exact NOE by Relaxation matrix Analysis) [4.3]. Either \( \sigma_{ij}^{\text{app}} \) (or \( I_{ij}^{\text{app}} \)) is calculated with the full relaxation matrix approach, or individual correction contributions from each neighbor spin \( k \) are obtained from the exact solutions of three-spin systems \( ijk \) and are then summed up. Importantly, this
method can also be applied to partially deuterated proteins. Understanding the impact of spin diffusion and its correction is very crucial and is discussed in detail in the following section.

4.3.1. Correction for spin diffusion

4.3.1.1. Full matrix approach

In the eNORA routine [4.3], the diagonal peak intensities derived from the NOESY spectra are fitted to mono-exponential decay functions to extract the auto-relaxation rate constants, \( \rho_i \) and \( \rho_j \), and the initial intensities, \( I_{ii}(0) \) and \( I_{jj}(0) \). Then, cross peak buildup curves are simulated with the full relaxation matrix approach given in equation 4.4. PDB coordinates of a previously known structure (such as a conventionally determined NMR structure or an X-ray structure) are required. Corrections for the intensities at each mixing time are derived from the simulation and applied to the experimental intensities. The corrected cross-peak buildup curves are fitted by using \( \rho_i \), \( \rho_j \), \( I_{ii}(0) \) and \( I_{jj}(0) \) as fixed input parameters and the cross-relaxation rate constants \( \sigma_{ij} \) and \( \sigma_{ji} \) as free variables (if the correction is applied to the apparent experimental cross-relaxation rate instead of the intensities \( \sigma_{ij} \) and \( \sigma_{ji} \) are modified accordingly). The quality of the fit is evaluated and \( \sigma_{ij} \) and \( \sigma_{ji} \) are converted into distance restraints \( r \) using equation 2.49 (or 2.54). A structure calculation is then performed with the new distance restraints. This new structure may be used again as an input for the next correction calculation and refinement of the structure.

4.3.1.2. Three-spin system approach

Instead of calculating the simulated intensities via the full relaxation matrix approach, individual corrections for each neighboring spin \( k \) obtained from the exact (numerical) solution of
three-spin systems $ijk$ are summed up. Three-spin build-up curves are simulated for all cases where proton $k$ is located within spheres of 5 - 10 Å radii centred at $i$ and $j$, using cross-relaxation rates predicted from the distances in a structure, and autorelaxation rates taken from experiment (when available) or also predicted. These numerically simulated intensities are then fitted with the two-spin equation; the resulting fitted cross-relaxation rate simulates the rate that one would observe in an experiment (if $i$, $j$ and $k$ were the only spins present), and is here referred to as the apparent rate $\sigma_{ij}^{\text{app}(k)}$. The contribution from each spin $k$ to spin diffusion is thus the difference of the apparent rate and the theoretical two-spin rate, $(\sigma_{ij}^{\text{app}(k)} - \sigma_{ij})$, and the overall apparent rate is the sum of the two-spin rate and all the three-spin contributions involving $k$ spins. The correction factor is thus:

$$p_{ij} = \frac{\sigma_{ij}}{\sigma_{ij} + \sum_k \left( \sigma_{ij}^{\text{app}(k)} - \sigma_{ij} \right)}$$  \hspace{1cm} (4.20)

We have shown that this approach is in agreement with the eNORA approach for a large range of molecular overall tumbling times and NOESY mixing times, where spin diffusion is clearly effective.

4.3.1.3. Spin diffusion in (partially) deuterated molecules

Almost all quantitative measurements of NOE buildups have been conducted on protonated samples. In practice, this approach is limited to molecules no larger than ca. 35 kDa. To bypass this limitation, samples with partial deuteration are used to reduce spectral overlap and relaxation. Quantitative observation of NOE buildups is also possible in deuterated and perdeuterated samples. However, even in samples as highly deuterated as 99 % for H$^\alpha$ and 95 % for other
carbon-bound protons, spin diffusion pathways through residual protons cannot be neglected. For example, in ubiquitin with an overall correlation time of 7.7 ns (measured at high concentration at 284 K), \( \rho_i \) is typically 2 s\(^{-1} \) and the largest \( \sigma_y \) are ca. 1.5 s\(^{-1} \). In Figure 4.2, fitting of equations 4.13 and 4.15 up to mixing times of 90 and 200 ms is shown. The approximations are good for most NOEs in the amide proton network without corrections for spin diffusion. An averaged overestimation of only 9 % in the rate translates into an error of 1 % in the averaged distance. However, the approximation breaks down for protons in non-consecutive residues in the \( \alpha \) helix and loops, where corrections of 10 to 50 % are required. A comparison of corrections for a perdeuterated and a protonated sample is shown in Figure 4.3.
Figure 4.2. A representative set of simulated NOE build-up curves of perdeuterated ubiquitin is shown during a 0.2 s mixing time. The green NOE build-up curves expressed as transfer functions $T_{ij}^{\text{NOE}}$ are calculated under the assumption of two-spin systems. Intensities simulated at the mixing times used in the analysis are calculated in presence of spin diffusion pathways via spins that are within 5 Å of both spins in the pair (red circles). Fits through these points assuming a two-spin system are shown as blue curves.
Figure 4.3. Impact of spin diffusion on the determination of NOE rate constants between amide spin pairs and the dependence on the NOE mixing time $\tau_{\text{mix}}$ and the overall correlation time $\tau_c$. The corrections which must be applied to apparent experimental cross-relaxation rate constants are shown for a perdeuterated a) and a protonated protein b). Representative spin pairs from ubiquitin are selected. Spins in a $\alpha$ helix separated by one, two and three residues are exemplified by spin pairs 27/28, 27/29 and 27/30, spins separated by one residue in a $\beta$ strand by spin pairs 15/16 and from different $\beta$ strands by spin pairs 2/15, and spins separated by two residues in a loop by spin pairs 58/60. The ratio of $\tau_c$ and $\tau_{\text{mix}}$ is fixed at $10^{-7}$. For the perdeuterated protein, the deuteration level is assumed to be 99 % for $\text{H}^\alpha$ and 95 % for all other non-exchangeable protons. Reproduced from reference 4.4.

In general, the distribution pattern of the residual protonation level is highly complex in a macromolecule. Popular deuteration schemes yield a deuteration between 70 and 90 % or methyl-reprotonation in otherwise deuterated samples. The problem can be significantly simplified if the calculation of the overall contribution is divided into individual contributions from 3-spin systems. The impact of the deuteration levels can be accounted for by scaling the corresponding contributions by the residual protonation level without restriction from non-uniformity in the
deuteration level. As a rule-of-thumb, it is recommended to maintain a constant product of the maximum NOE mixing time and the overall tumbling time of ca. $5 \times 10^{-10} \text{s}^2$ for deuterated samples, while for protonated samples the value is approximately $2.5 \times 10^{-10} \text{s}^2$.

4.3.2. Validation and selection of experimental build-ups

It is crucial to assess the quality of the NOEs. For example, the NOEs that produce unreliable cross-relaxation rates should only be interpreted conservatively or dismissed completely. One obvious benchmark is the correction factor $p$ that should be as close to 1 as possible. It is also important to assess the quality of the build-up curve per se. This check should be carried out at different stages of the overall procedure (for example, before or after peak intensity correction for spin diffusion). The quality of the build-up fits is jeopardized by two main contributions: Firstly, the validity of the assumptions made in equations 4.13 and 4.15 (this depends, for example, on the extent of spin diffusion or possible peak overlap); secondly, the signal-to-noise ratio. Different quality factors have been proposed to judge the agreement of the cross-peak build-up and the two-spin approximation. We initially used the following expression:

$$\chi = \frac{1}{\max \{I_{\text{exp}}\}} \sqrt{\frac{1}{N_{\text{mix}} - 1} \sum_{i=1}^{N_{\text{mix}}} \left( I_{\text{exp}} \left( \tau_{\text{mix}}(i) \right) - I_{\text{fit}} \left( \tau_{\text{mix}}(i) \right) \right)^2}$$  \hspace{1cm} (4.21)

where $N_{\text{mix}}$ is the number of mixing times and $I_{\text{exp}}$ and $I_{\text{fit}}$ are the experimental and back-predicted peak intensities, respectively. As a guideline, ‘good fits’ are obtained for $\chi < 0.08$ and ‘bad fits’ for $\chi > 0.15$, respectively.

eNORA calculates two other values for quality assessment of the fits. One is Pearson’s correlation coefficient $R^2$ and the other one $Q$: 
\[ R^2 = \frac{\sum_{i=1}^{N_{\text{mix}}} (I_{\text{exp}}(\tau_{\text{mix}}(i)) - T_{\text{exp}})(I_{\text{fit}}(\tau_{\text{mix}}(i)) - T_{\text{fit}})^2}{\sum_{i=1}^{N_{\text{exp}}} (I_{\text{exp}}(\tau_{\text{mix}}(i)) - T_{\text{exp}})^2 \sum_{i=1}^{N_{\text{fit}}} (I_{\text{fit}}(\tau_{\text{mix}}(i)) - T_{\text{fit}})^2} \]  
\[ Q = \frac{\sum_{i=1}^{N_{\text{mix}}} (I_{\text{exp}}(\tau_{\text{mix}}(i)) - I_{\text{fit}}(\tau_{\text{mix}}(i)))^2}{\sum_{i=1}^{N_{\text{exp}}} I_{\text{exp}}(\tau_{\text{mix}}(i))^2} \]  

In our experience, ‘good peaks’ obtained for the same data set have \( R^2 \) values larger than 0.8, which is a default value in eNORA. Note, however, that it is only a good criterion if the buildup is approximately linear as \( R^2 \) is a measure for the linear dependence between \( I_{\text{exp}} \) and \( I_{\text{fit}} \). \( Q \) is a variant of \( \chi \) where the normalization is modified.

For technical reasons, we find that an exact experimental cross-relaxation rate constant can only be obtained if the build-ups of both cross peaks caused by a spin pair \( i-j \) can be fitted (\textit{vide infra}). In that case, the two fitted values are combined to the experimental value as follows:

\[ \sigma_{ij}^{\text{exp}} = \sigma_{ji}^{\text{exp}} = -\sqrt{\sigma_{ij}^{\text{fit}} \sigma_{ji}^{\text{fit}}} \]  

The justification of equation 4.24 is given in equation 3.10.

4.3.3. Distance calculation

Effective distances, which absorb all motional effects, are obtained from equations 2.49 and 2.54. For the structure calculation (\textit{vide infra}), we translate \( \tau_{ij}^{\text{eff}} \) into upper and lower limits of distance restraints. If both magnetization pathways can be analysed following equation 3.10, the upper and lower distance bounds are both set to \( \tau_{ij}^{\text{eff}} \). If the NOE rate can be determined from one
pathway only, the lower and upper distance bounds are set to $0.85 r_{ij}^{\text{eff}}$ and $1.15 r_{ij}^{\text{eff}}$, respectively. This choice is based on a comparison with a highly accurate structure where the violations appear approximately symmetrical. The corresponding cross-relaxation rate constants are twice (lower distance limit) or half as large (upper distance limit) as the exact value as shown in Figure 4.5. Based on a very recent in-depth investigation, we recommend to set to limits to $0.80 r_{ij}^{\text{eff}}$ and $1.20 r_{ij}^{\text{eff}}$. It may be argued that in practice the error is expected to be symmetric in the cross-relaxation rate constant rather than in the effective distance. Such a choice would result in a non-symmetric tolerance for the upper and lower distance limits. The advantages of such limits and the generality of the bounds used for the GB3 data sets are subject of on-going research in our laboratory.

Figure 4.5. Relationship between cross-relaxation rate constants and the corresponding distances. The black curve indicates the rate constant corresponding to the true distance. The curves show rate constants resulting from addition of 15 % or subtraction of 15 % with respect to the true distance, respectively. The inset shows an expansion of the relationships for long distances.
In general, it appears very challenging to estimate experimental errors and thus a reasonable choice of upper and lower limits. The accuracy of the full matrix approach as such has been analysed, in particular the impact of the diagonalization in combination with experimental errors. Limitations of the method have been demonstrated but it is not straightforward to translate these results into rules for the choice of bounds.

4.3.4. Impact of motions

If motions are not explicitly accounted for in the analysis of $r_{ij}^{\text{eff}}$ as obtained from equations 2.49 and 2.53, the question arises as to what influence dynamics has on the experimental values. It may be desirable to neglect its impact or estimate the induced error in a ‘rigid structure’ analysis. In the following, the fluctuation of the vector connecting atoms or pseudo atoms (if a group of equivalent spins is present) is considered.

The amide proton-amide proton NOE order parameters due to fast motion have been estimated for ubiquitin. The calculation is based on the experimentally determined H^N-N order parameters obtained from relaxation measurements. The nitrogen atoms were assumed to be located on a rigid backbone and the amide protons to sample a cone with Gaussian distributed weights (depending on the excursion from the average orientation) consistent with the H^N-N order parameters. The results are plotted in Figure 4.6a and are rationalized by theoretical calculation shown in Figure 4.6b. As the large majority of the H^N-N order parameters is larger than 0.8, all NOE order parameters fall between 0.95 and 1.10 with the majority clustered within 3% deviation from 1. The reasons for this effect are twofold. Firstly, the distances between the
two protons involved in the NOE are larger than the H\textsuperscript{N}-N distances. Secondly, the angular and distal effects counteract to a large extent. Note also that the estimation constitutes an upper limit to the expected impact from fast motion because it is likely that the motions are in part correlated and thus reduce the effect. This suggests that the impact of fast motions can be approximately neglected for H-H spin pairs as long as the local H-X order parameters are larger than 0.5, which is usually fulfilled in globular proteins except for highly flexible tails and loops.

**Figure 4.6.** Relationship between order parameter of the NOE between backbone amide proton spins $i$ and $j$, $(S_{\text{HH}}^{\text{fast}})^2$, and the local order parameters of the H\textsuperscript{N}-N vectors of residues $i$ and $j$, $(S_{\text{NH}}^{\text{fast}})^2$. a) Fast-motion NOE order parameters versus the product of the corresponding local order parameters of ubiquitin determined experimentally at 288 K. Consecutive residues in $\alpha$ helices, $\beta$ sheets and loops are represented by open circles, and non-consecutive residues by filled circles, respectively. For large and small values of $(S_{\text{HH}}^{\text{fast}})^2$ the involved residues are indicated. b) Theoretical surface plot showing $(S_{\text{HH}}^{\text{fast}})^2$ versus the product of two corresponding $(S_{\text{NH}}^{\text{fast}})^2$ (having identical values) and the distance between the two cones in the interval from 2 to 5 Å. The two conformational scenarios yielding the most extreme values, cones opposing (top surface) or facing each other (bottom surface), are illustrated. Facing yields an order parameter smaller than 1, while opposing larger than 1. Reproduced from reference 4.5.
The situation is not as trivial if side-chain atoms are involved in the NOEs. Obviously, the assumption of Gaussian fluctuations around a single energy minimum conformation is an extreme simplification.

4.3.5. Validation of experimental distances

We measured amide proton-amide proton NOE build-ups with a series of 3D [$^{15}$N]-resolved HMQC-NOESY experiments on a perdeuterated ubiquitin sample. Then, we derived effective distances with equation 2.49 without applying any correction for spin diffusion. An excellent correlation is obtained with distances extracted from a high-resolution X-ray structure (Figure 4.7). The estimated experimental random error is ≈ 0.07 Å or on average ca. 2 % for distances up to 5 Å. This error is smaller than the pairwise r.m.s. deviation from corresponding distances extracted from high-resolution NMR or X-ray structures (pdb codes: 1D3Z and 1UBQ, both 0.24 Å). Remarkably, it is also smaller than the pairwise r.m.s.d. between the corresponding distances in the X-ray and NMR structures (0.15 Å).

The next question is whether distances obtained from eNOEs measured on protonated macromolecular samples with a much denser spin network are of similar quality. We measured amide proton-amide proton NOE build-ups with two series of 3D [$^{15}$N]-resolved HMQC-NOESY and 3D [$^{15}$N]-resolved NOESY-HSQC experiments on a deuterated GB3 sample, and a series of 3D [$^{15}$N]-resolved NOESY-HSQC experiments on a protonated GB3 sample. All slopes of linear regressions through correlation plots of any two sets deviate from 1 by less than 2 %. All correlation coefficients are larger than 0.95 and larger than 0.97 for distances derived from both cross peaks.
Figure 4.7. Correlation plot showing $H^N-H^N$ distances obtained from the X-ray structure 1UBQ with protons placed at ideal positions versus effective distances extracted from experimental cross-relaxation rates of perdeuterated ubiquitin without correction for spin diffusion. Distances between two spins in the $\beta$ sheet are red, $\alpha$ helix purple, and those involving one or two spins in a loop blue. Distances between two spins of consecutive (non-consecutive) residues in a secondary structural element are triangular (square). The slope of the black line is 1. Reproduced from reference 1.26.

Subsequently, we used a protonated GB3 sample to derive eNOE-based distance constraints between any proton types from a series of 3D $^{15}N,^{13}C$-resolved NOESY. Overall, 1092 build-up could be fitted (typically satisfying the criterion $\chi < 0.15$ with $\chi$ defined in equation 4.21). 562 of them constitute pairs of both transfer pathways of a specific spin system resulting in $562/2 = 281$
exact cross-relaxation rate constants. The remaining 530 do not have the corresponding counterpart and resulted in 530 less exact cross-relaxation rate constants. For the structure calculation (vide infra), H\textsuperscript{N}-H\textsuperscript{N} distances were calculated from various data sets and an average overall set was used which has a residue-averaged random error of only 0.06 Å moving 31 NOEs obtained from a single cross peak to the group with 0% error tolerance and yielding an additional 12 eNOEs (in total, 324 from both pathways/multiple H\textsuperscript{N}-H\textsuperscript{N} data sets, and 499 from one pathway or pairs involving methyl groups). The sequence-specific distribution of the restraints is shown in Figure 4.8.

**Figure 4.8.** Number of eNOE-derived distance restraints for GB3 versus residue number. The counts are classified as short-range (white), medium-range (gray), and long-range (black), where \( i \) and \( j \) are the residue numbers of the involved spins. Reproduced by from reference 4.6.
The cross-relaxation rates were corrected for spin diffusion and converted into effective distances under the assumption of isotropic molecular tumbling following equations 2.49 and 2.53. The determined effective distances absorb all motional effects. Figure 4.9 shows correlation plots between the experimental effective and predicted distances. The slope of the linear regression including all distances is 0.97, whereas it is 0.99 for backbone distances only. The slope drops as the protons are located further out in the side chains, which is attributed to motion, as stated already above. Pearson’s correlation coefficient for all distances for which both pathways could be evaluated is 0.92, while for the backbone only it is 0.98. Inclusion of distances calculated from only one cross peak lowers it to 0.84 for all distances and 0.96 for the backbone.

It can be concluded that the correlation between the experimental and true distances is very good. Taking into account that the reference structure was determined under different conditions and necessarily introduces an additional error from the shortcomings of a single conformer representation, the experimental eNOE data are of extraordinarily high precision. Since the quality factors $\chi$ for NOEs for which a side-chain proton was involved are comparable to those for backbone NOEs, the precision and accuracy of these distances are expected to be similar to those in the backbone. However, the correlation of the distances between spins in the side chains is not as good as those in the backbone. This apparent discrepancy is mainly attributed to the more pronounced side-chain motion, which is evidently insufficiently represented by the single-state X-ray reference structure.
Figure 4.9. Predicted versus experimental distances in GB3. Experimental distances were calculated under the assumption of isotropic molecular tumbling with a correlation time $\tau_c = 4.15$ ns at 298 K. Predictions are based on a X-ray structure with RDC-optimized backbone proton positions. The panels show the distances between (top left) two spins located in the backbone (bb); (top right) one in the backbone and one being a side-chain $\text{H}^{\beta(2,3)}/\text{Q}^\beta$; (bottom left) one in the backbone and one being another side-chain proton (sc, rest); and (bottom right) both in the side chain as indicated in the black boxes. Black lines indicate slope 1. Reproduced from reference 4.6.
5. Structure calculation

Structure calculation using the full-matrix approach can hardly be separated from the extraction of cross-relaxation rates from NOEs and has been touched on in the previous chapter. Since no consensus procedure has emerged yet, it is also nearly impossible to separate the biological context from the specific method. First, the most innovative examples are reviewed. Soon after the first structure calculations it has been recognized that some NOEs can only be explained by multiple conformations. The development of the calculation of multiple-conformation ensembles (sometimes referred to as sets) of structures rather than single structures is reviewed in the next section. In the last section, the approach to calculate an ensemble from eNOEs developed in our laboratory is discussed in more detail.

5.1. Structure calculation using the full-matrix approach

The first application of the full relaxation-matrix approach to 2D NOESY was probably the conversion of six cross-peak and four diagonal-peak intensities into six distances of the small molecule proflavine [5.1]. The distances were within 10% of those obtained from an X-ray crystal structure. This important work was followed by an increasing number of applications to increasingly more challenging systems. Most applications have been carried out on nucleic acids rather than proteins. The structural characteristics of RNA and DNA are more subtle and small differences often determine their function due to the lack of tertiary structure. In addition, the lower and more uneven proton density (3.4 neighbouring protons within a sphere of 3 Å in B-DNA versus 4.7 in a globular protein) drove the development with more urgency.
5.1.1. Nucleic acids

The first primitive approach towards macromolecular structure calculation was undertaken on nucleic acid fragments. The very first report dates back to 1985 when James et al. used the full-matrix approach to predict intensities from a standard DNA B-form and an energy-minimized DNA B-form structure obtained from AMBER. Both intensity sets were compared to experimental 2D NOESY spectra of the DNA octamer [d(GGAATTCC)]₂ recorded with four mixing times assuming different correlation times [5.2].

Figure 5.1. Left: Stereo view of the structure obtained after three cycles of IRMA starting from B-DNA. Right: Stacked plots of parts of the 2D NOE spectra of the DNA octamer at various mixing times. The strong peak belongs to the H5-H6 cross peak of cytidine C9. Taken from reference 4.2.

The first example of a direct structure calculation via exact NOEs is the use of the IRMA program to obtain the DNA octamer d(GCGTTGCG):d(CGCAACGC) with an estimated
correlation time of 1 ns in 1989 (see Figure 5.1) [4.2]. 167 cross-peak volume build-ups were obtained from a series of eight 2D \[^1\text{H},^1\text{H}\]-NOESY spectra with mixing times ranging from 5 to 200 ms. After 100-300 steps of steepest-descent restrained energy minimizations, the restrained MD calculations were performed for 40 ps. The averaged MD structures were used as updated structure input after another energy minimization. After three IRMA cycles convergence was achieved for canonical A- and B-form starting structures. Convergence criteria were either convergence of the experimental distances or of the r.m.s. deviation between different starting structures. Importantly, all starting structures converged towards the B-form.

Another pioneering application to nucleic acids was a structural refinement of the extrahelical adenosine tridecamer d(CGCAGAATTCGCG)\(_2\) with the MORASS program together with rMD calculations in AMBER [5.3]. Convergence was judged with the following percentage \(RMS\) figure and \(R\) factor (not to be confused with the relaxation matrix \(R\)):

\[
\%RMS = \left[ \frac{1}{N} \sum \left( \frac{I_{\exp} - I_{\text{calc}}}{I_{\text{calc}}} \right)^2 \right]^{1/2} \times 100
\] (5.1)

and

\[
R_{\text{factor}} = \frac{\sum |I_{\exp} - I_{\text{calc}}|}{\sum I_{\exp}}
\] (5.2)

The sum runs over all intensities (which could possibly also extend over different mixing times). The \(R\) factor criterion is analogous to that used in X-ray crystallography. However, the authors recommend the use of \(\%RMS\) because it is not dominated by short distances. A 2D NOESY with 150 ms mixing time yielded 258 constraints. A crystal structure shows the extrahelical adenosine looped out way from the duplex. However, the NMR data established that it stacks into the duplex. Importantly, the ISPA approach alone was not able to place the extrahelical adenosine.
Other $R$ factors have also been proposed:

$$
\tilde{R}_{\text{factor}} = \frac{\sum |I_{\text{exp}}^{1/6} - I_{\text{calc}}^{1/6}|}{\sum I_{\text{exp}}^{1/6}}
$$

(5.3)

This figure of merit relates the intensities to the coordinate space of the structure. The weak NOEs have again more weight than in the previous expression. The $R$ factor has also been calculated with a well potential where the flat bottom breadth is determined by experimental errors.

5.1.2. From peptide to proteins

As early as in 1986 exact distances derived from the full-relaxation matrix approach have been used in an additional force field in AMBER to analyse a complex of ristocetin pseudoglycon and Ac2-Lys-D-Ala-D-Ala [5.4].

The first protein structure obtained from the full relaxation-matrix approach with experimental data was that of a 18-residue peptide with an amino-acid sequence comprising the first zinc-finger like domain from the gag protein p55 of HIV in 1990 (see Figure 5.2) [5.5]. A series of five 2D [$^1$H,$^1$H]-NOESY spectra with mixing times ranging from 5 to 500 ms yielded 226 distance constraints. The DSPACE distance geometry program was used to generate initial structures in the standard manner. The cycle between generation of the $I$ and $R$ matrices was reiterated with the BKCALC and GNOE programs. Early cycles were repeated until the predicted and experimental spectra were visually identical. Later cycles produced a match between calculated and experimental build-up and decay curves. Additional refinement steps such as simulated annealing were finally applied.
More recently, many applications of a partial relaxation-matrix approach appeared in the field of structure calculation of ligand-protein complexes.

It must be noted that virtually in all studies distances were extracted by comparing cross-relaxation rate constants to those of NOEs with known distances. This procedure is correct if the reference vector has a similar value for the order parameter and a similar extent of spin diffusion as the vector under investigation. This is a strong simplification and not recommended for future studies. Instead, the overall correlation time should be determined.
5.2. Ensemble calculations using conventional NOE-distance restraints

The rationale behind ensemble calculation is that an NMR observable of the average structure is not equal to the average of the observables taken for each snapshot. For example, some distances in the previously mentioned ristocetin pseudo-aglycon/Ac2-Lys-D-Ala-D-Ala complex derived from the full relaxation-matrix approach were inconsistent with energy-minimized structures [5.4]. Another 10 ps MD run revealed two clusters of low-energy structures with different orientations of the F and G rings. It was concluded that the NOEs are averaged over both conformations.

In the following, approaches to generate ensembles of structures are outlined. Two main categories may be distinguished. The first introduces an additional force field or similar into MD simulations, called restrained MD or rMD. As such, it is a time-averaged approach. The second one collects all other approaches, which calculate ensembles in more direct ways, which are essentially conformational averages. Note that most of these approaches also employ rMD to some extent and a strict separation is somewhat arbitrary.

5.2.1. Restrained molecular dynamics simulation

In a first systematic approach to account for NOE averaging, a 20 ps rMD simulation has been run with the memory function as proposed in equation 2.58 with \( q = 3 \) [1.22] on the 74-residue tendamistat with 842 distance restraints [5.6]. For that purpose, the force field was replaced by a constructed force in order to avoid occasional large forces due to forth-power terms. The violations were shown to be reduced as compared to a conventional rMD run.
In a further developed approach, MD trajectories based on simultaneous time- and space Boltzmann-weighted averaging have been shown to yield a more appropriate description of experimental NOE data than single-molecule methods.

5.2.2. Direct structural ensemble calculation

The first protocol to directly extract multiple conformations from NOE data (and other NMR data) was MEDUSA (Multiconformational Evaluation of Distance information Using a Stochastically constrained minimization Algorithm) [5.7]. The protocol makes use of both distance restraints and anti distance restraints (that is, absence of a cross peak). First, a large set
of static structures is generated by fulfilling all anti distance restraints and a subset of the distance restraints. Then, a clustering procedure selects an ensemble of structures that fulfills all distance restraints. MEDUSA was applied to antamanide with 23 distance restraints, 108 anti distance restraints and scalar couplings. From 1176 initial structures, a considerable number of pairs adequately satisfied the experimental data.

Subsequently, more straightforward procedures were introduced. A 15-residue peptide corresponding to the N-terminal sequence of bovine pancreatic trypsin inhibitor (BPTI) destabilized by replacement of the two cysteines by serines was synthesized to mimic a folding intermediate [5.8]. As it is disordered and presumably cannot satisfy NOEs with a single conformation well, an ensemble-calculation protocol has been implemented. 93 initial build-up rates were converted into identical upper and lower distance restraints (that is, exact constraints) by calibration with the NOE between Hδ and He in tyrosine. After calculating initial structures by a standard procedure, 8-member ensembles were generated by performing energy minimization and molecular dynamics with $<r^{-3}>$ averaging.

$r^{-6}$-averaging was tested with X-PLOR 3.1 on an ensemble representations of GB3 (56 residues, 854 distance restraints) and ragweed allergen Amb t V (40 residues, 1031 distance restraints) for which synthetic distance restraints were generated [5.9]. Violations were significantly reduced upon using twin conformers instead of one structure and the correct variability was reproduced. It has also been recognized that the tightness of the upper and lower bounds critically determines the optimal number of structures to be used. Real NOE/scalar coupling data sets for the proteins interleukin 4 (130 residues, 1735 NOE and 27 hydrogen bond distance restraints) and interleukin 8 (71 residues, 1764 NOE and 116 hydrogen bond distance restraints) were analysed and their effective B factors were compared to previously determined
crystallographic $B$ factors [5.9]. While one structure appropriately represents the interleukin 4 ensemble (with a $B$ factor considerably lower than the X-ray analogue), twin conformers are appropriate for interleukin 8 (Figure 5.4). For the latter, the NMR and X-ray $B$ factors are similar. The difference is driven by two conformations in the loop comprising residues 16-22.

![Figure 5.4](image)

**Figure 5.4.** Comparison of the single-state (a) and two-state ensembles (b) of interleukin 8 generated from conventional NOEs. Taken from reference 5.9.

Clore and Schwieters resurrected the multiple-conformer ensemble calculation on the model proteins GB3 and ubiquitin by torsion angle simulated annealing and Cartesian space minimization in Xplor-NIH [5.10,5.11]. Major progress is achieved by the larger experimental data sets. Typically, the experimental input data comprised NOEs, scalar couplings ($J$) and
residual dipolar couplings (RDCs) measured in multiple alignment media. The minimized energy term is

$$E_{\text{total}} = E_{\text{NOE(only UBQ)}} + E_J + E_{\text{RDC}} + E_{\text{covalent geometry}} + E_{\text{nonbonded contacts}} + E_{\text{RAP}} + E_{\text{shape}}$$

The NOE energy term is a flat-bottom (accounting for uncertainties) quadratic harmonic well with back-calculated distances taken as \( r^6 \)-average. RDCs depend on the orientation of the vector spanned by two protons relative to a molecule-fixed frame and thus contain complementary information to the NOE input. A relative atomic position (RAP) term is introduced that prevents single members to stray too far from ensemble-averaged positions. It was applied to the C\(^\alpha\) atoms. It has the same form as the NOE term, with the uncertainties replaced by an allowed distance deviation. A molecular shape term prevents excessive rotation and deformation of single members. Cross-validation on ubiquitin and GB3 shows that no further improvement is achieved if more than two structures are used. \( S^2(\text{jump}) \) for backbone H\(^N\)-N vectors is usually larger than 0.8 with values as small as 0.3 for flexible residues. In GB3, extensive anticorrelated crankshaft motion is observed along the backbone.

An ensemble of ubiquitin was recalculated from NOEs (\( r^6 \) averaging) and large sets of RDCs with the EROS protocol (see Figure 5.5) \[5.12\]. The refinement with the GROMACS package was achieved by simultaneous application of NOE and RDC restraints on sub-ensembles of eight structures. The resulting ensemble with a mean backbone rmsd of 1.22 Å covers the complete spatial heterogeneity represented by 46 crystal structures. Each of these structures is within less than 0.8 Å backbone r.m.s. deviation from one NMR ensemble member. Since most of these structures are in complex with other molecules it was concluded that conformational selection is
sufficient to explain the molecular recognition dynamics. The authors conclude that part of the dynamics occurs in the ‘blind’ time window of nanoseconds to tens of microseconds. In a follow-up study, the 640-member ensemble termed ERNST (Ensemble Refinement for Native proteins using a Single alignment Tensor) with a backbone rmsd of 0.83 Å was obtained using ensemble MD simulations restrained by 2663 NOEs and 1971 H\textsuperscript{N}-N RDCs [5.13]. The structure calculations were carried out with the CHARMM27 force field in explicit solvent. To ensure that the structure of ubiquitin was well reproduced, the NOEs were restrained over pairs of structures. Finally, collective motions spanning the β strands separated by up to 15 Å were identified and characterized. These correlations are mediated in part by the hydrogen-bonding network and link molecular recognition sites.

**Figure 5.5.** 40 backbone traces of EROS ensemble of ubiquitin. Residues are colored by the amount of additional (supra-τ\textsubscript{c}) mobility as compared with the Lipari-Szabo order parameters \((S^\text{supra})^2 = (S^\text{EROS})^2/(S^\text{LS})^2\). Taken from reference 5.12.
5.3. *Multiple-state structural ensemble calculation using eNOEs*

The information content of upper (and lower) limit distance restraints derived from NOEs has been subject to theoretical studies. It is clear that tighter constraints than those usually used would lead to better defined structural ensembles. In particular, addition of lower limit restraints improve the definition further. It has also been shown that the sensitivity of a single NOE to the exact nature of conformation distributions is relatively low [5.14]. Instead, the strength of the eNOEs for defining structures and dynamics lies in the cumulative effect of multiple eNOEs. Due to the technical challenges outlined above, all structural ensembles published as of now are obtained from conventional NOEs (that is, single mixing-time measurements rather than series, and NOEs converted into semi-quantitative distance ranges) with one exception: Upper and lower distance limits derived from initial NOE build-up curves were used for an ensemble calculation of the linear peptide YQNPDGSQA [1.24]. As outlined in Chapter 4.3, we established a procedure to obtain effective distances of high precision from eNOEs in our laboratory. In what follows, a protocol for multiple-state structure calculation in CYANA [5.15,5.16] is presented and exemplified with the protein GB3. We use the term ‘multiple-state’ structure, instead of the previously used ‘multiple-conformer’ structure.

5.3.1. *Conventional structure calculation*

For the conventional structure calculation, 1953 upper distance limits calculated from the cross-peak intensities of the NOESY with a mixing time of $\tau_{\text{mix}} = 100$ ms resulted in 1041 constituted meaningful restraints. Additional experimental restraints were $^3J_{HN\alpha}$, $^3J_{HNC}$, and $^3J_{HNC\beta}$ scalar couplings, N-H$^N$ and C$^\alpha$-H$^\alpha$ RDCs, and angular restraints from $^{13}$C$^\alpha$ chemical shifts. Using
these restraints a standard structure calculation was performed with the software package CYANA [5.15,5.16] starting with 100 randomized conformers. Simulated annealing with 50'000 torsion-angle dynamics steps was applied and the 20 conformers with the lowest final target function values were selected. Small pairwise r.m.s. deviations of 0.47 Å for the backbone atoms and 0.87 Å for all heavy atoms, respectively, indicate good convergence of the structure calculation. The 9 structures with the lowest target functions are shown in Figure 5.7 on the left.

5.3.2. Multiple-state structure calculation protocol

For the multiple-state ensemble calculation, the set of effective distances presented in section 4.3.5. was used. The following error tolerances were chosen: ±0 %, ±15 %, and ±20 % (-1 Å per methyl group for lower limit) for distances calculated from both cross peaks/multiple Hα-Hα sets, one cross peak, and those involving two methyl groups, respectively. For NOEs involving at least one aromatic proton Hδ or Hε of Phe or Tyr, only an upper limit set (8 Å). In addition, the scalar couplings, RDCs and chemical shifts mentioned above were used. All of these observables are sensitive to motion on the millisecond timescale (the ‘slow’ NMR timescale).

As for the conventional structure calculation protocol, 100 conformers were calculated with the software package CYANA and the 20 conformers (here, 20 multi-state ensembles) with the lowest target function values were then used to represent the calculated structure. Ensembles encompassing 1 to 9 states of the entire protein were calculated simultaneously, using the same number of initial conformers and the same simulated annealing schedule as for the conventional structure calculation (see Figure 5.6). The squared differences between each eNOE-derived effective distance and the corresponding distances obtained by $r^{-6}$-averaging over the states are
minimized. Similarly, the $^{3}J$ coupling restraints and the RDC restraints were applied to the arithmetic mean of the corresponding quantities in the individual states. In addition, the angular restraints derived from $^{13}$C chemical shifts are required to be fulfilled and steric repulsion between atoms of different states was excluded.

![Figure 5.6](image)

**Figure 5.6.** Target functions (TF) values obtained from structure calculations versus the number of simultaneously optimized states (a-c) and their r.m.s. deviations (d). The overall TF is shown in (a), the contributions from the eNOEs in (b), and those from the RDC, $J$ coupling, van-der-Waals, $C^{\alpha}$ angular and bundling restraints in (c), respectively.
Due to the $r^{-6}$-dependency long distances contribute minimally to the NOE. This may result in an unphysically loose packing of the ensemble. Bundling restraints were applied in order to keep the individual structural states together in space as far as permitted by the experimental restraints. To this end weak upper distance bounds of 1.2 Å were applied to all distances between the same nitrogen and carbon atoms in different states. The weight of these bundling restraints was 100 times lower than for NOE upper distance bounds, except for the backbone atoms N, C$^\alpha$, C$'$ and C$^\beta$, for which a 10 times lower weight than for NOEs was used. This weight is such that the restraints can easily be overridden by the experimental restraints and it also ensures that the different $\chi_1$ rotamer states of the side chains are within the r.m.s. deviation without violating the bundling restraint.

The number of states necessary to describe the experimental data is not known a priori. Therefore, an array of structure calculations with the number of states varying from 1 to 9 was performed (see Figure 5.6). Among these ensembles the one with the minimal number of states that satisfies the experimental data well is selected as the appropriate representative. This ensemble with $X$ states is obtained if the three following criteria are fulfilled: (i) The target function drops significantly from state $(X-1)$ to $X$, (ii) the target function does not drop significantly anymore upon an increase of the number of states to $(X+1)$, and (iii) a jack-knife error estimation does not produce a lower-than-random target function for omitted eNOEs upon an increase of the number of states to $(X+1)$. Following these criteria, it appears that the ensemble with $X = 3$ states is an appropriate representation of the GB3 structure.
Figure 5.7. Heavy-atom structural representations of GB3 obtained from either the classical protocol with NOEs as experimental input, the classical protocol with eNOEs, or the ensemble-based protocol with eNOEs. **Left:** Bundle calculated with a classical protocol based on standard NOE measurements. Nine conformers are shown. **Middle:** Single-state bundle calculated with eNOEs. Nine conformers are shown. **Right:** 3 three-state ensembles obtained from eNOEs. The three most similar structures from each three-state conformer are grouped in gold, red and blue. Reproduced from reference 1.26.

9 members of the single-state bundle are shown in Figure 5.7 in the middle. The input data results in an extremely tight structure with a small backbone pairwise r.m.s. deviation of 0.11 Å and an all-heavy-atom r.m.s.d. of 0.60 Å only (compare to conventional bundle, left). Furthermore, the eNOE-based single-state NMR structure coincides closely with a highly accurate RDC-optimized X-ray structure with an r.m.s.d. of 0.57 Å for the backbone and 1.17 Å for all heavy atoms. However, the large target function value of 27.5 Å² resulting from many distance restraint violations indicates that the structure does not agree with the experimental data.
The large number of violations of experimental restraints can be attributed to the motion-averaged nature of the measured NOE, while the structure calculation protocol is based on a single static structure. In contrast, for the three-state ensemble shown on the right of Figure 5.7 the TF value is reduced to 9.9 Å².

Interestingly, the entire β sheet and some of the loops undergo conformational exchange between the three states in a concerted fashion. In contrast, the α helix appears to be decoupled from the conformational exchange of the β sheet. The backbone of the α helix shows also distinct structural states, but the correlation is weaker than for the β sheet and is localized to the residues that face the hydrophobic core.

5.3.2.1 Improvements to the protocol

In current studies, we use three modifications of the ensemble calculation protocol.

Firstly, the CYANA protocol is executed with an individual treatment of each methyl proton (CYANA command ‘expand = 1’). This approach has been shown to be more efficient than the use of pseudo atoms. Therefore, previous input distance restraints were scaled by $3^{1/6}=0.833$ per methyl group such that the corresponding cross-relaxation rate constant is a sum over all individual contributions. This is strictly true if the methyl motion is slow (slower than nanoseconds). Since there is fast rotation present as well, we added an additional tolerance of +/- 8.5 % to the upper/lower limit distance restraints.

Secondly, the previously used data set is extended by eNOEs that involve either methylene groups with chemically equivalent protons or chemically equivalent methyl groups in VAL and LEU. The apparent cross-relaxation rate constants are fitted to the same formulae as used for
single atoms or methyl groups and normalized to the equivalent of a superposition of contributions from all pairs of single atoms. Note that this is an approximation because the spins do not undergo fast exchange. Instead, the spectral peaks are superpositions of the individual buildup/decay curves. Therefore, all upper limits and lower distance limits were given an additional tolerance of 5 % in addition to the 0 % / 15 % for bidirectional / unidirectional eNOEs.

Thirdly, angular constraints from C\(^{\alpha}\) shifts improve convergence during the CYANA structure calculation, but are not desired to restrain the final structure due to their statistical rather than exact relationship. Therefore, the weight of the angular constraint contribution to the target function is increasingly reduced during the calculation (‘anneal_weight_aco’ is set to 1.0, 1.0, 0, 0). On the other hand, scalar couplings and RDCs are known to slow down convergence if they are fully active at the initial stage of the calculation due to local extrema. Therefore, their contribution weight to the target function is ramped up during calculation (CYANA command ‘anneal_weight_cco’ and ‘anneal_weight_rdc’ are set to 0, 0.5, 1, 1). This procedure renders the distances restraints computed from the eNOEs, which do not have individual local extrema, the main factors at the initial stage.

5.3.3. Cross-validation of the ensemble

To validate the three-state ensemble representation of GB3, a check of the self-consistency of the input data and a comparison with independently obtained data and were performed. The self-consistency checks were undertaken in two ways (Figure 5.8a). First, the eNOE-derived distances obtained from both cross peaks were arbitrarily changed according to normal distributions with standard deviations of 5, 10, and 15 %, or alternatively all eNOE-derived distances by 10 %,
respectively. The target function values of the corresponding structure calculations are considerably larger in all cases, for example by a factor of ca. two in the cases of the 10 % changes. In the second check, the eNOE data is cross-validated by a jack-knife error estimation. Ten structure calculations were run from which 10 % of the input distance restraints were randomly omitted (each NOE is omitted in only one of the calculations). Then, an overall target function was calculated by summing over the target functions calculated for the omitted data. This cross-validation target function tends to decrease with increasing number of states. The target function for the three-state ensemble is 40 % lower than the one for the single-state structure. This outcome indicates that the structure of the three-state ensemble obtained with the reduced experimental data set is close to the one calculated with the entire data set. Therefore, the ensemble is at least in part over-determined and self-consistent.

Independent checks for the three-state ensemble of GB3 may also be obtained by comparison of X-ray and NMR parameters that quantify spatial sampling. For example, the angular excursion from an average orientation of the backbone HN-N vectors can be quantified by order parameters $S^2$. Good agreement between those calculated from the ensemble and order parameters derived from RDCs measured under six alignment conditions was obtained (Figure 5.8b). The minimal number of states previously determined to represent the data well is also the minimal number that yields satisfying order parameters. Another example for a check constitutes a comparison between the obtained side-chain rotamer states and those in a high-resolution X-ray structure (here 1.1 Å, PDB code 1IGD). The NMR ensemble includes all the rotamer states observed in the crystal structures with the exception of residues 7 and 47. For residues 15, 21 and 35, the same two rotamer states as in the crystal structure are observed. Scalar couplings and RDCs as well as cross-correlated relaxation rates measured in liquid state are also in very good agreement with the
rotamer states of the structural ensemble including residues 7 and 47. Some inconsistencies with the X-ray structure may be due to different sample conditions such as crystalline/liquid state.

**Figure 5.8.** Cross-validation of the optimal number of states. **a)** The cross-validation target functions (TF) for three-state ensembles are shown. TFs obtained from a jack-knife procedure are shown in blue, and upon random alteration of the distances obtained from both cross peaks by 5, 10, and 15 % in yellow, orange, and red, respectively, and upon random alteration of all distances by 10 % in pink. **b)** Backbone H\textsuperscript{N}-N order parameters obtained for ensembles calculated with 2, 3, 4, 5, and 9 states versus the amino sequence of GB3 are shown. For comparison, order parameters obtained from RDCs are drawn in blue.
6. Transferred NOE

(Dr. Julien Orts)

The system of interest is a pair of ligands, A and B, which bind to the same target, T. We assume the following exchange scheme:

\[ AT + B \xrightleftharpoons[k_{21}]{k_{12}} A + BT \]  \hspace{1cm} (6.1)

For simplicity, T in the free form is not explicitly treated. It can be shown that for practical scenarios the inclusion of terms for free T does not alter the results.

We would like to describe the process with a combined Solomon and McConnell equation for the following magnetization vector

\[
\begin{pmatrix}
\langle I_{A,z} \rangle & \rightarrow \langle I_{A,0} \rangle \\
\langle I_{B,z} \rangle & \rightarrow \langle I_{B,0} \rangle \\
\rightarrow \text{Abound} I_{A,z} & \rightarrow \text{Abound} I_{A,0} \\
\rightarrow \text{Abound} I_{T,z} & \rightarrow \text{Abound} I_{T,0} \\
\rightarrow \text{Bbound} I_{B,z} & \rightarrow \text{Bbound} I_{B,0} \\
\rightarrow \text{Bbound} I_{T,z} & \rightarrow \text{Bbound} I_{T,0}
\end{pmatrix}
\]  \hspace{1cm} (6.2)

Each entry is of vector form again, describing the complete spin systems of the free and bound A and B, and the one of T either bound to A or to B.

Combining equations 2.30 and 2.32 gives the relevant \( R' = R + K \) matrix
\[
R' = \begin{pmatrix}
R_A + k_{21}[TB] & -k_{12}[B] & -k_{21}[A] \\
-k_{21}[TB] & R_A + k_{12}[TA] & R_{A,T} \\
-k_{12}[TA] & R_{T,TA} & R_{T,A} + k_{12}[B] \\
-k_{12}[B] & R_{T,B} & R_{T} + k_{21}[A] \\
\end{pmatrix}
\]

where \( R_A, R_B \) and \( R_T \) are the relaxation matrices of the spins of A, B and T with the dimensions corresponding to the number of spins in A, B and T, respectively. An additional feature is the inclusion of the concentrations of A, B, TA and TB written in rectangular parentheses. This is necessary because the concentrations of the spins may be different when they are located in different types of molecules. The symbol 1 indicates unity matrices with the same dimensions as the relaxation matrices they are added to.
7. Outlook

In summary, it is shown how highly precise and accurate NOEs can be measured (exact NOEs, eNOEs). It is relatively simple to record and evaluate the data provided sufficient experimental time is available. A simple conversion results in very exact averaged distances, which allow one to reveal even small effects. In particular, the eNOE is very sensitive to translational motion between (groups of) spins. The quality of the experimental data is sufficient to calculate multiple-state ensembles, which can be regarded as a discrete three-dimensional representation of protein motion.

At this point, our approach is still under development. We are currently exploring the potential of the method in answering biological questions. Nevertheless, there remains a number of open questions and challenges. Answering and overcoming them would pose major advances for the method. In the following, a list of the most important points is given.

7.1. Future challenges

7.1.1. Experimental

Can a complete excitation profile be achieved in the heavy-atom dimension of a NOESY experiment? In our experiments used so far, eNOEs that involve pseudo protons in the aromatic rings were only analyzed in a semi-quantitative manner because carbon excitation and inversion pulses with rectangular profiles and their counterparts after the carbon evolution period cannot efficiently excite a frequency range broader than 100 ppm. To a lesser extent, the same effect is one of the reasons for a less rigid choice of the upper and lower distance restraints if only one
cross peak can be evaluated. One obvious solution is to run a second build-up series with a shifted carrier frequency. However, this is not attractive since it would double the measurement time. It is conceivable that more dedicated pulses could be used to cover the full carbon frequency range, possibly in combination with different time increments (analogous to the simultaneous use of $^{15}$N and $^{13}$C evolution). Such a procedure may even produce two different spectral widths and resolutions for the two carbon frequency ranges.

**How large can the systems studied be?** Our detailed studies of eNOEs from the model proteins GB3 and ubiquitin with molecular weights of 6.5 and 8.5 kDa have been extended to cyclophilin A with 16 kDa. Larger systems pose three fundamental challenges.

First, large systems have large overall correlation times, which cause faster transverse relaxation rates. As a consequence, magnetization is lost during the pulse sequence. Longitudinal relaxation is slower so that the interscan delays must be increased. However, NOESY experiments belong to a group of experiments that can be used for very large systems because they rely on the transfer of longitudinal magnetization that is more favourable than transverse magnetization in terms of relaxation, and the transfer is more efficient for increasing overall tumbling times. Theoretical considerations and experiments demonstrate that NOESY can be recorded with large deuterated proteins of several hundred kilodaltons.

The second limitation is caused by increased peak overlap for large systems. The problem is particularly pressing with respect to the diagonal peaks. Overcoming this hurdle is an active research field and holds promise for the future. Recording NOESYs with additional dimensions, such as 4D HXQC-NOESY-HXQC type pulse schemes would separate the two proton resonances by the shifts of two heavy atoms. In general, approaches to achieve a higher signal-to-
noise ratio also lead to a higher resolution. Another way to make the experiments more efficient (and to achieve higher resolution) is to use sparse sampling. Note that simple Fourier transformation is not the optimal method for processing the data. Good spectral quality for doubly $^{13}$C-resolved experiments has been demonstrated with systems of up to ca. 80 kDa for sparse sampling in combination with multidimensional decomposition (MDD). A challenge which is particularly prominent for NOESY is the large dynamic range of the peak intensities (typically up to three orders of magnitude). Among other approaches, compressed sensing (CS) reconstruction of undersampled 3D NOESY promises to alleviate this problem substantially.

A third fundamental limitation is that for high molecular weight systems, it is more difficult to obtain a concentrated solution, and thus the longer it takes to acquire a mixing-time series of NOESY experiments. Our experiments are typically carried out with samples at ca. 2 mM protein concentration, which is much higher than would normally be used for present-day NMR structural work, and is simply unattainable for many proteins and nucleic acids.

**eNOEs in solid-state NMR?** So far, efficient and exact determination of internuclear distances by solid-state NMR in uniformly labelled samples has proven difficult. Techniques such as rotational resonance ($R^2$) for homonuclear $^{13}$C-$^{13}$C spin pairs and rotational echo double resonance (REDOR) for heteronuclear $^{13}$C/$^{19}$F or $^{15}$N/$^{19}$F spin pairs have been used to quantify distances between isolated spin pairs. The uncertainty is smaller than 1 Å. However, it would be desirable to have an analogue to liquid-state NOESY that delivers distances throughout the entire molecule. In such experiments, the longitudinal $^{13}$C magnetization may be exchanged while the proton decoupler is switched off ($Proton Driven Spin Diffusion$, PDSD) or operates at a weak field ($Dipolar-Assisted Rotational Resonance$, DARR). Due to the strong dipolar truncation
typical of first-order recoupling methods, small couplings cannot be detected. Instead, second-order recoupling sequences which exhibit weaker truncation effects are used. Other experiments such as CHHC and NHHC make use of $^1$H-$^1$H transfer. For routine structure calculations, the results of several types of experiments are used. Typically, the presence of a cross peak can be interpreted in terms of a distance up to 5 Å. The determination of distances in a more quantitative manner is hampered by the fact that a powder-averaged spectrum cannot be described exactly by rate equations.

7.1.2. Ensemble calculation

**How many eNOEs are needed?** Since cross-validation procedures indicate that the experimental data set of GB3 is in part over-determined, the question arises as to how much data is needed to obtain at least two distinct states in an ensemble. For example, we reduced the experimental input for the GB3 ensemble calculation by omitting the $J$ couplings and RDCs. Interestingly, the r.m.s. deviation remains virtually identical for the three-state ensemble. However, extraction of long-range correlations is less straightforward, although it is still possible. Therefore, it is recommended to measure a small set of $J$ couplings and/or RDCs in addition to the eNOEs. On the other hand, we did not use eNOEs involving pseudoatoms of methylene groups, only conventional upper limits for aromatics, and we used very conservative restraints for methyl groups. We are currently working on the quantitative use of all these eNOEs.

**What is the best choice for the upper and lower limits?** So far, we have used limits that are symmetric about the distance restraints. More intuitively, the limits should be symmetric about
the cross-relaxation rate constants. We are currently investigating the generality of our choice of numeric tolerances if only one cross peak for a given spin pair could be evaluated.

**Is there a perfect cross-validation?** Complete self-consistent over-determination will probably never be achieved. Typically, well-defined regions of a molecule may be partially overdetermined, while other parts remain underdetermined. Although many cross-validation procedures have been proposed, an ideal self-consistency check is very difficult to establish. An inferential structure determination protocol may be used to account for the incomplete definition of an NMR structure by calculation of probability distributions.

**How can we represent segmental optimal numbers of states?** In our approach, the entire molecule is represented by a uniform number of states. However, it is likely that flexible parts of the molecule require more states and rigid ones less states for a proper representation. For example, one may wonder what happens in a segment with two very distinct, equally populated states if a three-state ensemble is postulated.

As mentioned above, inferential structure determination may turn out to be the protocol of choice.

**Can we correctly incorporate the time scales?** In virtually all ensemble refinement protocols, all NMR observables are averaged in a uniform manner, irrespective of the time windows they are sensitive to. To make things even worse, NOE rates average differently over short and long time scales, and the practical choice of averaging ($r^6$ versus $r^3$) seems rather heuristic in most publications.
Can we detect allosteric mechanisms? Efficient propagation of local perturbations through the protein must be mechanically mediated by coupling elements. Taking an ensemble point of view, allostery is based on the redistribution of populations of the states. In principle, such motions should be detectable with the eNOE method.

7.1.3. Transferred NOE

Can we drive the complex structure calculation by the full-matrix approach? Structure calculation makes use of the derivate of the energy related to the $R'$ matrix. As of now, convergence of the structure calculation has been hindered by the complexity of this derivative and the full-matrix approach is mainly used for structure refinement. (Dr. Julien Orts)
8. Appendix

The Master equation

The stochastic Hamiltonians are expressed in terms of irreducible tensor operators of rank 2, \( T_m \), and stationary random functions given by second order spherical harmonics, \( F_m \):

\[
H_1(t) = \sum_{m=-2}^{2} T_m(t) F_m(t)
\]  

(A1.1)

Transformation of \( H_1(t) \) into the interaction frame gives:

\[
H_{1\text{int}}(t) = \sum_{m,n} T_m t_n F_m(t) e^{i\omega_m t}
\]  

(A1.2)

with

\[
[H_0, T_m^n] = \omega_m^n T_m^n
\]  

(A2)

\( T_m^n \) and \( \omega_m^n \) are the eigenfunctions and the eigenvalues of \([H_0, .]\). Note that many different conventions are used throughout the literature. Here, the conventions of reference [2.2] are chosen with the exception that the \( r \) dependence is absorbed into the stationary random function in order to conserve the time-dependence of \( r \). The physical constants are collected into the irreducible tensor operator by introducing the following factor dipolar interactions:

\[
A_{\text{D}(i,j)} = \sqrt{3} \frac{\mu_0}{10} \frac{\gamma^2 \hbar}{4\pi \langle r_{\text{rapid}}^3 \rangle^3}
\]  

(A3)
Since the polarizing magnetic field is along the z-axis, \( \vec{B}_0 \) reduces to \((0, 0, B_0)^T\). Transverse operators are expressed by raising and lowering operators defined as:

\[
I_{i,\pm} = I_{i,x} \pm iI_{i,y}
\]

(A4)

For the dipolar Hamiltonian of spins \( i \) and \( j \), \( T_m \) and \( F_m \) are \((a = D(i,j))\)

\[
T^{D(i,j)}_0 = -\frac{A_D}{\sqrt{6}} \left( 4I_{i,z}I_{j,z} - \left( I_{i,+}I_{j,-} + I_{i,-}I_{j,+} \right) \right)
\]

(A5.1)

\[
T^{D(i,j)}_{\pm 1} = \pm A_D \left( I_{i,z}I_{j,\pm} + I_{i,\pm}I_{j,z} \right)
\]

(A5.2)

\[
T^{D(i,j)}_{\pm 2} = -A_D I_{i,\pm}I_{j,\pm}
\]

(A5.3)

\[
F^{D(i,j)}_m(t) = \left( \frac{r_{ij}^{\text{rigid}}}{r_{ij}(t)} \right)^3 \sqrt{4\pi} Y_{2m}^*(\theta_i(t), \phi_i(t))
\]

(A6)

\( \theta_i \) and \( \phi_i \) are the polar angles defining the orientation of the \( r \) vector connecting spins \( i \) and \( j \).

Transformation of \( T^{D(i,j)}_m \) into the interaction frame gives terms with \( n = 1,2,3 \):

\[
T^{1,D(i,j)}_0 = -\frac{A_D}{\sqrt{6}} 4I_{i,z}I_{j,z}
\]

(A7.1)

\[
T^{2,D(i,j)}_0 = \frac{A_D}{\sqrt{6}} I_{i,+}I_{j,-}
\]

(A7.2)

\[
T^{3,D(i,j)}_0 = \frac{A_D}{\sqrt{6}} I_{i,-}I_{j,+}
\]

(A7.3)

\[
T^{1,D(i,j)}_{\pm 1} = \pm A_D I_{i,\pm}I_{j,\pm}
\]

(A7.4)
\[ T_{\pm 1}^{2D(i,j)} = \pm A_D I_{ij} \pm I_{j,i} \]  
(A7.5)

\[ T_{\pm 2}^{1D(i,j)} = A_D I_{i,i} \pm I_{j,j} \]  
(A7.6)

and

\[ \omega_{0}^{1D(i,j)} = 0 \]  
(A8.1)

\[ \omega_{0}^{2D(i,j)} = \omega_i - \omega_j \]  
(A8.2)

\[ \omega_{0}^{3D(i,j)} = -(\omega_i - \omega_j) \]  
(A8.3)

\[ \omega_{\pm 1}^{1D(i,j)} = \pm \omega_i \]  
(A8.4)

\[ \omega_{\pm 1}^{2D(i,j)} = \pm \omega_j \]  
(A8.5)

\[ \omega_{\pm 2}^{1D(i,j)} = \pm (\omega_i + \omega_j) \]  
(A8.6)

In the derivation of equation 2.17, the following properties are used:

\[ F_m^a(t) \propto Y_{2m}^*(\theta_a(t), \varphi_a(t)) = (-1)^m Y_{2m}(-\theta_a(t), -\varphi_a(t)) \propto (-1)^m F_{-m}^a(t) \]  
(A9)

In the derivation of equation 2.18, the following property was used:

\[ \text{Trace}(A[B,C]) = \text{Trace}([A,B]C) \quad \text{for any operators } A, B, C \]  
(A10)
Non-degenerate and degenerate transitions in NOESY

In the following, some explicit calculations are shown for the evaluation of the relaxation matrix $R$ in equation 2.21. First, some useful spin operator commutation rules are recalled.

$$\left[ I_{i,\pm}, I_{i,\mp} \right] = \pm I_{i,\pm} \quad (A11)$$

$$\left[ I_{i,+}, I_{i,-} \right] = 2I_{i,z} \quad (A12)$$

$$\left[ I_{i,x}, I_{j,\mu} \right] = 0 \quad \mu = x, y, z, +, - \quad (A13)$$

$$[AB,C] = [A,C]B + A[B,C] \quad \text{for any operators } A, B, C \quad (A14)$$

The following relationships can easily be verified (for example, by means of the Pauli matrices).

$$I_{i,\pm}I_{i,\mp} = \pm \frac{1}{2} I_{i,z} \quad (A15)$$

$$I_{i,+}I_{i,-} = \frac{1}{4} \quad (A16)$$

$$I_{i,\pm}I_{i,\mp} = \pm \frac{1}{2} I_{i,z} \quad (A17)$$

$$I_{i,\mp}I_{i,z} = \pm \frac{1}{2} I_{i,\pm} \quad (A18)$$

A complete evaluation of equations 2.19.1 and 2.19.2 for $\langle b \rangle = \langle I_{i,z} \rangle$ is provided in Tables A1-A5. Some examples for $\langle b \rangle = \langle 2I_{i,z}I_{j,z} \rangle$ are shown in Table A9. It is assumed that the high-temperature approximation holds and $\rho_0 \propto H_0$ nulls all double commutators. A dipolar
interaction with $a = D(i,j)$ and $a' = D(i,j)$ leads to non-degenerate and additional degenerate auto-correlated relaxation pathways listed in Tables A1 and A2, respectively. Dipolar cross-correlated relaxation between $a = D(i,j)$ and $a' = D(i,k)$ and vice versa for non-degenerate transition is treated in Table A3, and for degenerate transitions in Tables A4 ($\omega_j = \omega_k$) and A5 ($\omega_i = \omega_j$). For $\langle b \rangle = \langle 2I_{i,z}I_{j,z} \rangle$, the non-degenerate transition cases for dipolar auto-correlated relaxation are shown in Table A6. Generalization to all other cases is trivial. Summation of all terms in Table A1 whose double commutator yields $I_{i,z}$ produces the auto-relaxation rate constants $R_{ij}^D$. The same procedure produces the cross-relaxation rate constant $\sigma_{ij}$ when the double commutators yield $I_{j,z}$. Inspection of Table A2 shows that dipolar auto-correlated relaxation is identical if non-degenerate transitions are included. Similar considerations with Table A3 demonstrate that cross-correlated relaxation creates two- and three-spin order. Non-degenerate transitions add pathways to zero-quantum transitions (see Tables A4 and A5). It is noted that this effect cannot be represented with the basis chosen in equation 2.23.

**Table A1.** Evaluation of relaxation pathways with $a = D(i,j)$ and $a' = D(i,j)$ for non-degenerate transitions and $\langle b \rangle = \langle I_{i,z} \rangle$.

<table>
<thead>
<tr>
<th>$\omega_m^{\alpha,\alpha}$</th>
<th>$(-1)^n$</th>
<th>factor</th>
<th>commutator</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>$\frac{8}{3} A_{D(i,j)}^2$</td>
<td>$[I_{i,z}, I_{j,z}, [I_{i,z}, I_{j,z}, I_{i,z}^+]] = [I_{i,z}, I_{j,z}, 0]$ = 0</td>
</tr>
<tr>
<td>$\omega_i$</td>
<td>-1</td>
<td>$-A_{D(i,j)}^2$</td>
<td>$[I_{i,z}, I_{j,z}, [I_{i,z}, I_{j,z}, I_{j,z}^+]] = [I_{i,z}, I_{j,z}, I_{i,z}^+] = [I_{j,z}, I_{i,z}^+, I_{j,z}^+] = 2I_{j,z}I_{j,z}$</td>
</tr>
<tr>
<td>$\omega_j$</td>
<td>-1</td>
<td>$-A_{D(i,j)}^2$</td>
<td>$[I_{i,z}, I_{j,z}, [I_{i,z}, I_{j,z}, I_{i,z}^+]] = [I_{i,z}, I_{j,z}, I_{i,z}^+] = [I_{j,z}, I_{i,z}^+, I_{j,z}^+] = 2I_{i,z}I_{j,z}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$[I_{i,z}, I_{j,z}, [I_{i,z}, I_{j,z}, I_{j,z}^+]] = [I_{i,z}, I_{j,z}, 0]$ = 0</td>
</tr>
</tbody>
</table>
\[ \omega_j - \omega_j \], \[ \omega_j + \omega_j \], \[ \omega_j - \omega_j \]

Table A2. Evaluation of additional relaxation pathways with \( a = D(i,j) \) and \( a' = D(i,j) \) for degenerate transitions and \( \langle b \rangle = \langle I_{iz} \rangle \).

<table>
<thead>
<tr>
<th>( \omega_j )</th>
<th>( \omega_j + \omega_j )</th>
<th>( \omega_j - \omega_j )</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-1)</td>
<td>(-A^{2}_{D(i,j)})</td>
<td>(-A^{2}_{D(i,j)})</td>
</tr>
</tbody>
</table>

\[ \left[ I_{iz}, I_{jz} \right] = \left[ I_{iz}, I_{jz} \right] = 0 \]

<table>
<thead>
<tr>
<th>( \omega_j )</th>
<th>( \omega_j + \omega_j )</th>
<th>( \omega_j - \omega_j )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \omega_j )</td>
<td>( A^{2}_{D(i,j)} )</td>
<td>( \frac{1}{6} A^{2}_{D(i,j)} )</td>
</tr>
</tbody>
</table>

\[ \left[ I_{iz}, I_{jz} \right] = \left[ I_{iz}, I_{jz} \right] = 0 \]

\[ \langle b \rangle = \langle I_{iz} \rangle \]

\[ \langle b \rangle = \langle I_{iz} \rangle \]

\[ \left[ I_{iz}, I_{jz} \right] = \left[ I_{iz}, I_{jz} \right] = 0 \]

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\[ \left[ I_{iz}, I_{jz} \right] = \left[ I_{iz}, I_{jz} \right] = 0 \]

\[ \left[ I_{iz}, I_{jz} \right] = \left[ I_{iz}, I_{jz} \right] = 0 \]
\[
\begin{array}{|c|c|c|}
\hline
0 & 1 & -2/3 A_{D(i,j)}^2 \\
\hline
0 & 1 & -2/3 A_{D(i,j)}^2 \\
\hline
\omega_i = \omega_j & -1 & -A_{D(i,j)}^2 \\
\hline
\omega_i = \omega_j & -1 & -A_{D(i,j)}^2 \\
\hline
\end{array}
\]

\[
\begin{align*}
0 & 1 \quad -2/3 A_{D(i,j)}^2 \\
\omega_i = \omega_j & -1 \quad -A_{D(i,j)}^2 \\
\omega_i = \omega_j & -1 \quad -A_{D(i,j)}^2 \\
\end{align*}
\]

\[
\begin{align*}
[I_{i,z} I_{j,z}, I_{i,-} I_{j,-}, I_{i,z}, I_{j,z}] &= [I_{i,z} I_{j,z}, I_{i,-} I_{j,-}] = \\
[I_{i,z}-I_{i,+} I_{j,z}, I_{i,-} I_{j,-}] &= \\
-I_{i,-} I_{j,z} + I_{i,-} I_{j,-} = & \frac{1}{2} I_{i,-} I_{j,-} - \frac{1}{2} I_{i,-} I_{j,z} = 0 \\
\end{align*}
\]

\[
\begin{align*}
I_{i,z} I_{j,z} + I_{i,-} I_{j,-} = & \frac{1}{2} I_{i,-} I_{j,-} - \frac{1}{2} I_{i,-} I_{j,z} = 0 \\
\end{align*}
\]

\[
\begin{align*}
I_{i,z} I_{j,z} + I_{i,-} I_{j,-} = & \frac{1}{2} I_{i,-} I_{j,-} - \frac{1}{2} I_{i,-} I_{j,z} = 0 \\
\end{align*}
\]

\[
\begin{align*}
I_{i,z} I_{j,z} + I_{i,-} I_{j,-} = & \frac{1}{2} I_{i,-} I_{j,-} - \frac{1}{2} I_{i,-} I_{j,z} = 0 \\
\end{align*}
\]

Table A3. Evaluation of cross-correlated relaxation pathways with $a = D(i,j)$ and $a' = D(i,k)$ and vice-versa for non-degenerate transitions and $\langle b \rangle = \langle I_{i,z} \rangle$.

<table>
<thead>
<tr>
<th>$\omega_{m}^{a,a'}$</th>
<th>$(-1)^m$</th>
<th>factor</th>
<th>commutator</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 &amp; 1 &amp; $8/3 A_{D(i,j)} A_{D(i,k)}$ &amp; $[I_{i,z} I_{j,z}, I_{i,-} I_{j,-}] = [I_{i,z} I_{j,z}, 0] = 0$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 &amp; 1 &amp; $8/3 A_{D(i,j)} A_{D(i,k)}$ &amp; $[I_{i,z} I_{j,z}, I_{i,-} I_{j,k}] = [I_{i,z} I_{j,z}, 0] = 0$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\omega_i$ &amp; -1 &amp; $-A_{D(i,j)} A_{D(i,k)}$ &amp; $[I_{i,z} I_{j,z}, I_{i,-} I_{j,k}] = [I_{i,z} I_{j,z}, I_{i,-} I_{j,k}]$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\omega_i$ &amp; -1 &amp; $-A_{D(i,j)} A_{D(i,k)}$ &amp; $[I_{i,z} I_{j,z}, I_{i,-} I_{j,k}] = [I_{i,z} I_{j,z}, I_{i,-} I_{j,k}]$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\omega_i$ &amp; -1 &amp; $-A_{D(i,j)} A_{D(i,k)}$ &amp; $[I_{i,z} I_{j,z}, I_{i,-} I_{j,k}] = [I_{i,z} I_{j,z}, I_{i,-} I_{j,k}]$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
\[\begin{align*}
\omega_i & \quad -1 \quad -A_{D(i,j)} A_{D(i,k)} \quad \left[ I_{i_z} I_{j_z} I_{k_z} \left\{ I_{i_z} I_{j_z} I_{k_z} - I_{i_z} I_{j_z} I_{k_z} \right\} \right] = \left[ I_{i_z} I_{j_z} I_{k_z} - I_{i_z} I_{j_z} I_{k_z} \right] \\
& \quad = 2 I_{i_z} I_{j_z} I_{k_z} = \frac{1}{2} A_{D(i,j)} A_{D(i,k)} 
\end{align*}\]

Table A4. Evaluation of additional cross-correlated relaxation pathways with \(a = D(i,j)\) and \(a' = D(i,k)\) and vice-versa for degenerate transitions with \(\omega_j = \omega_k\) and \(\langle b \rangle = \langle I_{i_z} \rangle\).

<table>
<thead>
<tr>
<th>(\omega_m^{a,a'})</th>
<th>((-1)^n)</th>
<th>factor</th>
<th>commutator</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\omega_j = \omega_k)</td>
<td>-1</td>
<td>(-A_{D(i,j)} A_{D(i,k)})</td>
<td>([ I_{i_z} I_{j_z} I_{k_z} - I_{i_z} I_{j_z} I_{k_z} ] = 2 I_{i_z} I_{j_z} I_{k_z} = \frac{1}{2} A_{D(i,j)} A_{D(i,k)} )</td>
</tr>
<tr>
<td>(\omega_j = \omega_k)</td>
<td>-1</td>
<td>(-A_{D(i,j)} A_{D(i,k)})</td>
<td>([ I_{i_z} I_{j_z} I_{k_z} - I_{i_z} I_{j_z} I_{k_z} ] = 2 I_{i_z} I_{j_z} I_{k_z} = \frac{1}{2} A_{D(i,j)} A_{D(i,k)} )</td>
</tr>
<tr>
<td>(\omega_j = \omega_k)</td>
<td>-1</td>
<td>(-A_{D(i,j)} A_{D(i,k)})</td>
<td>([ I_{i_z} I_{j_z} I_{k_z} - I_{i_z} I_{j_z} I_{k_z} ] = 2 I_{i_z} I_{j_z} I_{k_z} = \frac{1}{2} A_{D(i,j)} A_{D(i,k)} )</td>
</tr>
<tr>
<td>(\omega_j = \omega_k)</td>
<td>-1</td>
<td>(-A_{D(i,j)} A_{D(i,k)})</td>
<td>([ I_{i_z} I_{j_z} I_{k_z} - I_{i_z} I_{j_z} I_{k_z} ] = 2 I_{i_z} I_{j_z} I_{k_z} = \frac{1}{2} A_{D(i,j)} A_{D(i,k)} )</td>
</tr>
<tr>
<td>(\omega_j = \omega_k)</td>
<td>-1</td>
<td>(-A_{D(i,j)} A_{D(i,k)})</td>
<td>([ I_{i_z} I_{j_z} I_{k_z} - I_{i_z} I_{j_z} I_{k_z} ] = 2 I_{i_z} I_{j_z} I_{k_z} = \frac{1}{2} A_{D(i,j)} A_{D(i,k)} )</td>
</tr>
<tr>
<td>(\omega_j + \omega_j)</td>
<td>1</td>
<td>(A_{D(i,j)} A_{D(i,k)})</td>
<td>([ I_{i_z} I_{j_z} I_{k_z} - I_{i_z} I_{j_z} I_{k_z} ] = 2 I_{i_z} I_{j_z} I_{k_z} = \frac{1}{2} A_{D(i,j)} A_{D(i,k)} )</td>
</tr>
<tr>
<td>(\omega_j + \omega_j)</td>
<td>1</td>
<td>(A_{D(i,j)} A_{D(i,k)})</td>
<td>([ I_{i_z} I_{j_z} I_{k_z} - I_{i_z} I_{j_z} I_{k_z} ] = 2 I_{i_z} I_{j_z} I_{k_z} = \frac{1}{2} A_{D(i,j)} A_{D(i,k)} )</td>
</tr>
<tr>
<td>(\omega_j + \omega_j)</td>
<td>1</td>
<td>(A_{D(i,j)} A_{D(i,k)})</td>
<td>([ I_{i_z} I_{j_z} I_{k_z} - I_{i_z} I_{j_z} I_{k_z} ] = 2 I_{i_z} I_{j_z} I_{k_z} = \frac{1}{2} A_{D(i,j)} A_{D(i,k)} )</td>
</tr>
<tr>
<td>(\omega_j + \omega_j)</td>
<td>1</td>
<td>(A_{D(i,j)} A_{D(i,k)})</td>
<td>([ I_{i_z} I_{j_z} I_{k_z} - I_{i_z} I_{j_z} I_{k_z} ] = 2 I_{i_z} I_{j_z} I_{k_z} = \frac{1}{2} A_{D(i,j)} A_{D(i,k)} )</td>
</tr>
<tr>
<td>(\omega_j - \omega_j)</td>
<td>1</td>
<td>(\frac{1}{6} A_{D(i,j)} A_{D(i,k)})</td>
<td>([ I_{i_z} I_{j_z} I_{k_z} - I_{i_z} I_{j_z} I_{k_z} ] = 2 I_{i_z} I_{j_z} I_{k_z} = \frac{1}{2} A_{D(i,j)} A_{D(i,k)} )</td>
</tr>
<tr>
<td>(\omega_j - \omega_j)</td>
<td>1</td>
<td>(\frac{1}{6} A_{D(i,j)} A_{D(i,k)})</td>
<td>([ I_{i_z} I_{j_z} I_{k_z} - I_{i_z} I_{j_z} I_{k_z} ] = 2 I_{i_z} I_{j_z} I_{k_z} = \frac{1}{2} A_{D(i,j)} A_{D(i,k)} )</td>
</tr>
<tr>
<td>(\omega_j - \omega_j)</td>
<td>1</td>
<td>(\frac{1}{6} A_{D(i,j)} A_{D(i,k)})</td>
<td>([ I_{i_z} I_{j_z} I_{k_z} - I_{i_z} I_{j_z} I_{k_z} ] = 2 I_{i_z} I_{j_z} I_{k_z} = \frac{1}{2} A_{D(i,j)} A_{D(i,k)} )</td>
</tr>
<tr>
<td>(\omega_j - \omega_j)</td>
<td>1</td>
<td>(\frac{1}{6} A_{D(i,j)} A_{D(i,k)})</td>
<td>([ I_{i_z} I_{j_z} I_{k_z} - I_{i_z} I_{j_z} I_{k_z} ] = 2 I_{i_z} I_{j_z} I_{k_z} = \frac{1}{2} A_{D(i,j)} A_{D(i,k)} )</td>
</tr>
</tbody>
</table>
Table A5. Evaluation of additional cross-correlated relaxation pathways with $a = \mathrm{D}(i,j)$ and $a' = \mathrm{D}(i,k)$ and vice-versa for degenerate transitions with $\omega_i = \omega_j$ and $\langle b \rangle = \langle I_{i,z} \rangle$.

<table>
<thead>
<tr>
<th>$\omega_m^{n,a}$</th>
<th>(-1)$^m$</th>
<th>factor</th>
<th>commutator</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>$-2/3 \ A_{\mathrm{D}(i,j)}^2 A_{\mathrm{D}(i,k)}$</td>
<td>$[I_{i,z} I_{j,z}, [I_{i,z} I_{k,z}, I_{i,z}]] = [I_{i,z} I_{j,z}, -I_{i,z} I_{k,z}] = I_{i,z} I_{j,z} I_{k,z}$</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>$-2/3 \ A_{\mathrm{D}(i,j)}^2 A_{\mathrm{D}(i,k)}$</td>
<td>$[I_{i,z} I_{j,z}, [I_{i,z} I_{k,z}, I_{i,z}]] = [I_{i,z} I_{j,z}, -I_{i,z} I_{k,z}] = I_{i,z} I_{j,z} I_{k,z}$</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>$-2/3 \ A_{\mathrm{D}(i,j)}^2 A_{\mathrm{D}(i,k)}$</td>
<td>$[I_{i,z} I_{j,z}, [I_{i,z} I_{k,z}, I_{i,z}]] = [I_{i,z} I_{j,z}, -I_{i,z} I_{k,z}] = I_{i,z} I_{j,z} I_{k,z}$</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>$-2/3 \ A_{\mathrm{D}(i,j)}^2 A_{\mathrm{D}(i,k)}$</td>
<td>$[I_{i,z} I_{j,z}, [I_{i,z} I_{k,z}, I_{i,z}]] = [I_{i,z} I_{j,z}, -I_{i,z} I_{k,z}] = I_{i,z} I_{j,z} I_{k,z}$</td>
</tr>
</tbody>
</table>

Table A6. Evaluation of relaxation pathways with $a = \mathrm{D}(i,j)$ and $a' = \mathrm{D}(i,j)$ for non-degenerate transitions and $\langle b \rangle = \langle 2I_{i,z} I_{j,z} \rangle$.

<table>
<thead>
<tr>
<th>$\omega_m^{n,a}$</th>
<th>(-1)$^m$</th>
<th>factor</th>
<th>commutator</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>$8/3 \ A_{\mathrm{D}(i,j)}^2$</td>
<td>$[I_{i,z} I_{j,z}, [I_{i,z} I_{j,z}, I_{i,z}]] = [I_{i,z} I_{j,z}, 0] = 0$</td>
</tr>
<tr>
<td>$\omega_i$</td>
<td>-1</td>
<td>$-A_{\mathrm{D}(i,j)}^2$</td>
<td>$[I_{i,z} I_{j,z}, [I_{i,z} I_{j,z}, 2I_{i,z} I_{j,z}]] = [I_{i,z} I_{j,z}, (2I_{i,z} I_{j,z} + 0)] = 2I_{i,z} I_{j,z}$</td>
</tr>
<tr>
<td>$\omega_j$</td>
<td>-1</td>
<td>$-A_{\mathrm{D}(i,j)}^2$</td>
<td>$[I_{i,z} I_{j,z}, [I_{i,z} I_{j,z}, 2I_{i,z} I_{j,z}]] = [I_{i,z} I_{j,z}, (2I_{i,z} I_{j,z} + 0)] = 2I_{i,z} I_{j,z}$</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>$\omega_j$</th>
<th>$-1$</th>
<th>$-A_{D(i,j)}^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\omega_i + \omega_j$</td>
<td>$1$</td>
<td>$A_{D(i,j)}^2$</td>
</tr>
<tr>
<td>$\omega_i - \omega_j$</td>
<td>$1$</td>
<td>$\frac{1}{6} A_{D(i,j)}^2$</td>
</tr>
<tr>
<td>$\omega_i + \omega_j$</td>
<td>$1$</td>
<td>$A_{D(i,j)}^2$</td>
</tr>
<tr>
<td>$\omega_i - \omega_j$</td>
<td>$1$</td>
<td>$\frac{1}{6} A_{D(i,j)}^2$</td>
</tr>
</tbody>
</table>

\[
\begin{align*}
I_{i,z}I_{j,z} & \left[ I_{i,z}I_{j,z} + 2I_{i,z}I_{j,z} \right] = I_{i,z}I_{j,z} \left[ (I_{i,z}+2I_{i,z}I_{j,z})I_{j,z} + I_{i,z} \left[ I_{j,z} + 2I_{i,z}I_{j,z} \right] \right] \\
& = I_{i,z}I_{j,z} \left( 0 - 2I_{i,z}I_{j,z}I_{j,z} \right) = I_{i,z}I_{j,z} - \frac{1}{2} I_{j,z} \\
& = I_{i,z}I_{j,z} = \frac{1}{2} I_{j,z}
\end{align*}
\]

\[
\begin{align*}
I_{i,z}I_{j,z} & \left[ I_{i,z}I_{j,z} + 2I_{i,z}I_{j,z} \right] = I_{i,z}I_{j,z} \left[ (I_{i,z}+2I_{i,z}I_{j,z})I_{j,z} + I_{i,z} \left[ I_{j,z} + 2I_{i,z}I_{j,z} \right] \right] \\
& = I_{i,z}I_{j,z} \left( 0 - 2I_{i,z}I_{j,z}I_{j,z} \right) = I_{i,z}I_{j,z} - \frac{1}{2} I_{j,z} \\
& = I_{i,z}I_{j,z} = \frac{1}{2} I_{j,z}
\end{align*}
\]

\[
\begin{align*}
I_{i,z}I_{j,z} & \left[ I_{i,z}I_{j,z} + 2I_{i,z}I_{j,z} \right] = I_{i,z}I_{j,z} \left[ (I_{i,z}+2I_{i,z}I_{j,z})I_{j,z} + I_{i,z} \left[ I_{j,z} + 2I_{i,z}I_{j,z} \right] \right] \\
& = I_{i,z}I_{j,z} \left( 0 - 2I_{i,z}I_{j,z}I_{j,z} \right) = I_{i,z}I_{j,z} - \frac{1}{2} I_{j,z} \\
& = I_{i,z}I_{j,z} = \frac{1}{2} I_{j,z}
\end{align*}
\]

\[
\begin{align*}
I_{i,z}I_{j,z} & \left[ I_{i,z}I_{j,z} + 2I_{i,z}I_{j,z} \right] = I_{i,z}I_{j,z} \left[ (I_{i,z}+2I_{i,z}I_{j,z})I_{j,z} + I_{i,z} \left[ I_{j,z} + 2I_{i,z}I_{j,z} \right] \right] \\
& = I_{i,z}I_{j,z} \left( 0 - 2I_{i,z}I_{j,z}I_{j,z} \right) = I_{i,z}I_{j,z} - \frac{1}{2} I_{j,z} \\
& = I_{i,z}I_{j,z} = \frac{1}{2} I_{j,z}
\end{align*}
\]

\[
\begin{align*}
I_{i,z}I_{j,z} & \left[ I_{i,z}I_{j,z} + 2I_{i,z}I_{j,z} \right] = I_{i,z}I_{j,z} \left[ (I_{i,z}+2I_{i,z}I_{j,z})I_{j,z} + I_{i,z} \left[ I_{j,z} + 2I_{i,z}I_{j,z} \right] \right] \\
& = I_{i,z}I_{j,z} \left( 0 - 2I_{i,z}I_{j,z}I_{j,z} \right) = I_{i,z}I_{j,z} - \frac{1}{2} I_{j,z} \\
& = I_{i,z}I_{j,z} = \frac{1}{2} I_{j,z}
\end{align*}
\]

\[
\begin{align*}
I_{i,z}I_{j,z} & \left[ I_{i,z}I_{j,z} + 2I_{i,z}I_{j,z} \right] = I_{i,z}I_{j,z} \left[ (I_{i,z}+2I_{i,z}I_{j,z})I_{j,z} + I_{i,z} \left[ I_{j,z} + 2I_{i,z}I_{j,z} \right] \right] \\
& = I_{i,z}I_{j,z} \left( 0 - 2I_{i,z}I_{j,z}I_{j,z} \right) = I_{i,z}I_{j,z} - \frac{1}{2} I_{j,z} \\
& = I_{i,z}I_{j,z} = \frac{1}{2} I_{j,z}
\end{align*}
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\begin{align*}
I_{i,z}I_{j,z} & \left[ I_{i,z}I_{j,z} + 2I_{i,z}I_{j,z} \right] = I_{i,z}I_{j,z} \left[ (I_{i,z}+2I_{i,z}I_{j,z})I_{j,z} + I_{i,z} \left[ I_{j,z} + 2I_{i,z}I_{j,z} \right] \right] \\
& = I_{i,z}I_{j,z} \left( 0 - 2I_{i,z}I_{j,z}I_{j,z} \right) = I_{i,z}I_{j,z} - \frac{1}{2} I_{j,z} \\
& = I_{i,z}I_{j,z} = \frac{1}{2} I_{j,z}
\end{align*}
\]

\[
\begin{align*}
I_{i,z}I_{j,z} & \left[ I_{i,z}I_{j,z} + 2I_{i,z}I_{j,z} \right] = I_{i,z}I_{j,z} \left[ (I_{i,z}+2I_{i,z}I_{j,z})I_{j,z} + I_{i,z} \left[ I_{j,z} + 2I_{i,z}I_{j,z} \right] \right] \\
& = I_{i,z}I_{j,z} \left( 0 - 2I_{i,z}I_{j,z}I_{j,z} \right) = I_{i,z}I_{j,z} - \frac{1}{2} I_{j,z} \\
& = I_{i,z}I_{j,z} = \frac{1}{2} I_{j,z}
\end{align*}
\]
The Wigner rotation element

The Wigner element is

\[ D^l_{m'm} (\alpha, \beta, \gamma) = e^{-im'\alpha} d^l_{m'm} (\beta) e^{-im'\gamma} \]  

(A19)

where Wigner's d-matrix elements are

\[ d^l_{m'm} (\beta) = \sum_s (-1)^{s-m+s} \sqrt{(l+m)!(l-m')!(l+m')!} \frac{2l+2s}{2l+2m-2s-2} \left( \cos \frac{\beta}{2} \right)^{2l+2m-2s-2} \left( \sin \frac{\beta}{2} \right)^{m'+m+2s} \]  

(A20)

The Wigner rotation elements are related to the spherical harmonics as:

\[ D^l_{m'0} (\alpha, \beta, \gamma) = \left( \frac{4\pi}{2l+1} \right)^{\frac{1}{2}} Y^*_{lm} (\beta, \alpha) \]  

(A21)

\[ D^l_{0m} (\alpha, \beta, \gamma) = (-1)^{m} \left( \frac{4\pi}{2l+1} \right)^{\frac{1}{2}} Y^*_{lm} (\beta, \gamma) \]  

(A22)

\[ D^l_{m'm} (\alpha, \beta, \gamma) = D^l_{m'm} (\gamma, -\beta, -\alpha) \]  

(A23)
9. References


10. Glossary

Abbreviations:

Amb  ambrosia
AMBER  assisted model building with energy refinement
B factor  Debye-Waller factor
BPTI  bovine pancreatic trypsin inhibitor
CHARMM  chemistry at Harvard macromolecular mechanics
CHHC  solid-state experiment probing $^1$H-$^1$H magnetization exchange indirectly by $^{13}$C spins
CS  compressed sensing
CSA  chemical shift anisotropy
CSI  chemical shift isotropy
CYANA  combined assignment and dynamics algorithm for NMR applications
DARR  dipolar-assisted rotational resonance
DG  distance geometry
DNA  deoxyribonucleic acid
eNOE  exact nuclear Overhauser enhancement or effect
eNORA  exact NOE by relaxation matrix analysis
gag  group-specific antigen
GARP  globally optimized alternating phase
GB3  third immunoglobulin binding domain of protein G
Gly  glycine
HMOC  heteronuclear multiple-quantum correlation experiment
HSQC  heteronuclear single-quantum correlation experiment
HXQC  HMQC or HSQC
INEPT  insensitive nuclei enhanced by polarization transfer
IRMA  iterative relaxation matrix approach
ISPA  isolated spin pair approximation
Leu   leucine
Lys   lysine
MD    molecular dynamics
MDD   multidimensional decomposition
MEDUSA multiconformational evaluation of distance information using a stochastically constrained minimization algorithm
MM    molecular mechanics
NHHC  solid-state experiment probing $^1$H-$^1$H magnetization exchange indirectly by $^{15}$N and $^{13}$C spins
NMR   nuclear magnetic resonance
NOE   nuclear Overhauser effect or enhancement
NOESY nuclear Overhauser enhancement spectroscopy
PDB   protein data bank
Phe   phenylalanine
ppm   parts per million
PDSD  proton driven spin diffusion
R$^2$ rotational resonance
RAP   relative atomic position
RDC   residual dipolar coupling
REDOR rotational echo double resonance
rMD   restrained molecular dynamics
r.m.s.d. root-mean-square deviation
RNA   ribonucleic acid
ROE   rotating-frame Overhauser enhancement or effect
ROESY rotating-frame Overhauser enhancement spectroscopy
STD   saturation transfer difference
TF    target function
TPPI  time proportional phase increment
trNOE  transferred nuclear Overhauser enhancement or effect

Tyr  tyrosine

Val  valine

Symbols:

\( A \)  matrix describing the unperturbed motion of a spin system

\( A_{D(i,j)} \)  collected constants of dipolar interaction between spins \( i \) and \( j \)

\( B_0 \)  polarizing field vector

\( C_3 \)  three-fold rotational symmetry

\( C^{aa'}(.) \)  correlation function relating interaction mechanisms \( a \) and \( a' \)

\( d_{m'm}^l(.) \)  Wigner's d-matrix elements

\( D \)  dipolar interaction

\( D_{m'm}^l(.) \)  Wigner rotation elements

\( D_R \)  diagonalized relaxation matrix

\( Deut \)  \( D_2O \) level in per cent

\( E_X \)  energy term of constraint type \( X \)

\( F_m \)  stationary random functions expressed in second order spherical harmonics

\( \hbar \)  Planck’s constant divided by \( 2\pi \)

\( H_0 \)  stationary Hamiltonian

\( H_1(.) \)  stochastic Hamiltonian

\( i \)  pseudo proton number of a group of equivalent spins in vector form

\( I(.) \)  intensity matrix

\( I_i \)  spin operator of nucleus \( i \)

\( I_{i,\pm} \)  raising and lowering operators of spin \( i \)

\( J_{ij} \)  scalar coupling constant between spins \( i \) and \( j \)
\( J_m^{aa'}(.) \) spectral density function relating interaction mechanisms \( a \) and \( a' \)

\( k_{ex} \) exchange rate

\( K \) frame transformation operator (only in equation 2.14)

\( K \) matrix describing the exchange rates (in all cases other than equation 2.14)

\( K_{\text{NOE}} \) NOE force constant

\( N_{\mu\nu} \) population numbers of a two-spin \( \frac{1}{2} \) system in state \( \mu\nu \)

\( N_i \) number of magnetically equivalent spins \( \bar{i} \)

\( N_{\text{mix}} \) number of mixing times

\( P_2(.) \) second order Legendre polynomial

\( p_{ij} \) correction factor for cross-relaxation rate between spins \( i \) and \( j \)

\( Q \) quality factor

\( \bar{r}_{ij}(.) \) vector connecting nuclei \( i \) and \( j \)

\( R \) relaxation matrix

\( R' \) sum of \( R \) and \( K \)

\( R^2 \) Pearson's correlation coefficient

\( R^a \) relaxation rate constant due to interaction mechanism \( a \)

\( R_{\text{factor}} \) \( R \) factor

\( (S_{\text{exp}}^{a',\text{exp}})^2 \) squared experimental order parameter

\( (S_{\text{fast}}^{a',\text{fast}})^2 \) squared fast motion order parameter

\( t \) time

\( T_m \) spin operators expressed in second rank irreducible tensor operators

\( T_{\text{NOE}} \) magnetization transfer function for an NOE

\( U_R \) diagonalization matrix containing relaxation matrix eigenvectors

\( V_{ij}^{\text{NOE}} \) NOE term in force field
transition probability between eigenstates involving a change $x$ in the total spin of
by flippling spin(s) $Y$
atom position
second rank spherical harmonics
vector containing elements $\alpha_i$ in the Ernst notation of a population operator
fractional part of the magnetization that has recovered during the interscan delay
gyromagnetic ratio of the proton
relaxation superoperator
longitudinal cross-correlated relaxation rate constant between interactions $a$ and $a'$
steady-state NOE enhancement
contribution to dipolar autorelaxation due to spins $i$ and $j$ in longitudinal multi-
spin order
permeability in vacuum
density operator
equilibrium density operator
autorelaxation rate constants of $I_i, 4I_{i,z}I_{j,z}I_{k,z}$
standard deviation of peak intensities
cross-relaxation rate constant between spins $I_i$ and $I_j$
overall isotropic tumbling correlation time
effective correlation times
internal correlation time
memory function decay time
NOESY mixing time
quality factor
Larmor frequency of nucleus $i$
\( \omega_m^\nu \) \hspace{1cm} \text{differences between the eigenfrequencies of } H_0 \\
\Omega_i^{\text{CSI}} \hspace{1cm} \text{isotropic part of the chemical shielding tensor of nucleus } i \\
\%RMS \hspace{1cm} \text{percentage root-mean-square figure}