Conformational Dynamics in a Seven Transmembrane Protein Anabaena Sensory Rhodopsin Probed by Solid State NMR

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CONFORMATIONAL DYNAMICS IN A SEVEN TRANSMEMBRANE PROTEIN ANABAENA SENSORY RHODOPSIN PROBED BY SOLID STATE NMR

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Solid state NMR spectroscopy can be used to probe internal motions in membrane proteins in a lipid environment. In this study the site-specific measurements of the dipolar order parameters are reported and combined with the spin-lattice in the rotating frame (R\textsubscript{1ρ}) relaxation measurements carried out under fast magic angle spinning conditions in a seven transmembrane (TM) protein *Anabaena* Sensory Rhodopsin (ASR). Surprisingly, it was found that both the well-defined transmembrane regions and the mainly unstructured intramembrane loops and turns undergo restricted submicrosecond time scale motions corresponding to order parameters between 0.9 and 1.0. In contrast, the experimentally determined R\textsubscript{1ρ} relaxation rates vary by an order of magnitude between the TM and exposed fragments, indicating the presence of intermediate time scale motions that dominate the relaxation pathways for the exposed regions. Using a simple model that assumes a single exponential autocorrelation function, the dominant timescales of stochastic motions are estimated. Motions were found to be on the order of few nanoseconds for the TM helices, and tens to hundreds of nanoseconds for the extracellular B-C and F-G loops. These slower time scales may be associated with collective motions in these regions.
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List of Abbreviations

7TM    seven trans-membrane helical
ASR    Anabaena Sensory Rhodopsin
ASRT   Anabaena Sensory Rhodopsin Transducer
B₀     static magnetic field
CP     cross polarization
CSA    chemical shift anisotropy
DIPSHIFT dipolar chemical shift
DSS    2,2-dimethyl-2-silapentane-5-sulfonic acid
GB1    β1 immunoglobulin binding domain of protein G
HH     Hartmann-Hahn
MAS    magic angle spinning
NMR    nuclear magnetic resonance
PAS    principle axis system
ppm    parts per million
R₁ρ    transverse relaxation rate in the rotating frame
RF     radiofrequency
rms    root mean squared
SSNMR  solid state nuclear magnetic resonance
T-MREV transverse-Mansfield, Rhim, Elleman and Vaughan
UCN    uniformly $^{13}$C, $^{15}$N-labeled
ZF-TEDOR Z-Filtered Transferred Echo Double Resonance
Chapter 1 Introduction to Solid State Nuclear Magnetic Resonance of Membrane Proteins

1.1 Solid State Nuclear Magnetic Resonance in Structural Biology

Membrane proteins participate in the majority of life processes and perform numerous functions. For example, most extracellular stimuli experienced by the cell are first sensed by membrane receptors, which change their conformation and pass the signal inside the cell. The ability of membrane proteins to undergo conformational changes implies their high intrinsic flexibility which is essential to the protein function. However, the determination of structure and conformational dynamics of membrane proteins continues to pose a challenge for the traditional tools in structural biology, solution NMR and x-ray crystallography. Membrane proteins are amphiphilic and must be kept in a lipid environment to avoid denaturation. This poses a significant challenge to solubilisation for solution NMR studies and to crystallization for x-ray studies.

In response to the challenges associated with membrane protein structure determination solid state nuclear magnetic resonance (SSNMR) has recently emerged as a technique that allows for the determination of the structure of membrane proteins within a lipid environment. Until recently, experimental limitations have prevented the study of large proteins using SSNMR. Within the last decade these technical limitations have largely been overcome and a number of membrane protein structures have been determined.\textsuperscript{1–4} As with solution NMR, multidimensional magic angle spinning (MAS) SSNMR techniques have been used to probe other properties of the protein such as solvent accessible surface, conformational dynamics and oligomeric state. The solvent accessible surface has been probed by using hydrogen/deuterium exchange experiments.\textsuperscript{5,6} Conformational dynamics have been characterized roughly by measuring the
intensities of cross peaks observed in the through-space multidimensional chemical shift correlation experiments.\textsuperscript{7–9} In soluble proteins, more detailed descriptions of conformational dynamics have been provided by analysis of lineshapes of motionally narrowed anisotropic interactions, chemical shift anisotropies and heteronuclear dipolar couplings\textsuperscript{7,9–12} and by measurements of the longitudinal and transverse relaxation rates.\textsuperscript{13–15}

1.2 \textit{Anabaena} Sensory Rhodopsin

Recently our group has performed a series of SSNMR studies on a seven alpha-helical membrane protein \textit{Anabaena} Sensory Rhodopsin (ASR), a recently discovered microbial photosensor from cyanobacterium \textit{Anabaena} sp. PCC 7120.\textsuperscript{16} ASR has a typical rhodopsin architecture of a seven-helical (7TM) bundle, with retinal bound to lysine on the seventh helix (helix G) through the Schiff base.\textsuperscript{17,18} It initiates a unique phototransduction cascade, started by the absorption of light by retinal, which subsequently isomerizes from all-trans to 13-cis conformation, causing structural rearrangements within the protein, and modulating its binding affinity with its soluble cytoplasmic transducer (ASRT).\textsuperscript{16,17} Interactions between ASRT and DNA may regulate the expression of several proteins responsible for photosynthesis and circadian clock in \textit{Anabaena},\textsuperscript{19} thus providing a convenient mechanism for a single protein colour sensing.\textsuperscript{17,20–22}

Our group has previously determined that ASR forms trimers in lipids, with each monomer containing a rigid transmembrane core formed by seven helices. While seven helices form a similarly organized rigid transmembrane core of ASR in both crystals\textsuperscript{23} and in lipids,\textsuperscript{4} its extra-membranous loop regions are less structurally defined. Reduced NMR cross peak intensities of the residues in the loops (discussed in Chapter 3) and elevated B-factors in the crystal structure\textsuperscript{17} for the residues at the protein-solvent interface point to their flexibility. These
residues have exchangeable backbone amides,\textsuperscript{4,18} which again points at weaker intrahelical hydrogen bonds and potentially higher degree of flexibility within the exchangeable fragments. Hydrogen/deuterium exchange measurements under illumination indicates large changes in the water accessible surface of the protein, consistent with large conformational rearrangements, mostly on the cytoplasmic side.\textsuperscript{24} Finally, chemical shift assignments revealed double shifts for residues M41, F42, A91, M92, I95, K153, Y165, (Figure 1.1) indicating slow conformational exchange on the cytoplasmic interface.\textsuperscript{25}

![Figure 1.1](image)

**Figure 1.1** Amino acid sequence, secondary structure and spectroscopic assignments of ASR. Transmembrane helices are represented by rectangles. Assigned residues are shown in green. Residues shown in blue color have double sets of peaks.

Presently our group’s previous work is extended to determine the conformational dynamics of ASR using SSNMR. Dipolar chemical shift correlation spectroscopy (DIPSHIFT) is used to measure site-specifically the backbone dipolar order parameters and the magnitude of transverse spin-spin relaxation rates (R\textsubscript{1ρ}) to measure the site-specific motional correlation time. The data indicate that both the transmembrane and the extra-membranous solvent exposed regions undergo only small amplitude local motions. From the data it is estimated that, on average, the transmembrane regions are subjected to motions on a time scale of 3-30 ns, while
several loop regions experience slower dynamics in the hundreds of nanoseconds range. This approach can be extended to study the conformational dynamics in other membrane proteins.

1.3 Introduction to Solid State Nuclear Magnetic Resonance

The general concepts of Nuclear Magnetic Resonance (both solution and solid state) are explained at length in the reference books referred to in references 26–28. The main concepts in Solid State Nuclear Magnetic Resonance that will be drawn upon here come from the reference book “Multidimensional Solid State NMR and Polymers” by Schmidt-Rohr and Spiess.29

Nuclei composed protons (of spin ½ particles) and neutrons (no spin) can often have a non-zero angular momentum. The nuclei most commonly studied in NMR experiments of biological molecules are 1H, 13C and 15N all of which have a total spin of ½. When a spin ½ nucleus is placed in a static magnetic field its projection onto the axis of the magnetic field (assigned to be the z-axis by convention) is quantized into two discrete energy states. This is called the Zeeman interaction and is described by the following Hamiltonian:

\[ H_0 = -\hbar \gamma B_0 I_Z, \] (1.1)

where \( B_0 \) is the strength of the external magnetic field, \( \gamma \) is the gyromagnetic ratio, \( \hbar \) is the Planck’s constant and \( I_Z \) is the nuclear spin operator with eigenvalues of \( +\frac{1}{2} \) and \( -\frac{1}{2} \) for a spin \( \frac{1}{2} \) nucleus. At thermal equilibrium the lower energy state has a higher population with the ratio between populations related to temperature factor, \( k_B T \), by the Boltzmann distribution.

\[ \frac{N_{1/2}}{N_{-1/2}} = \exp \left( \frac{\hbar \gamma B_0}{k_B T} \right) \] (1.2)

The population difference between the two spin states causes the sample to have a magnetic moment that is aligned with the static magnetic field along the z-axis. The direction of
the magnetic moment can be rotated by surrounding the sample with a radio frequency coil that
generates a time dependent magnetic field perpendicular to the static magnetic field. If the
frequency of the time dependent magnetic field is equal to or very close to the Larmor frequency
of the nuclei, $\omega_0$, then the magnetic moment of the sample can only be rotated into the plane
perpendicular to the static magnetic field. The Larmor frequency is given by the energy level
difference between the two nuclear spin states.

$$\omega_0 = \frac{\Delta E}{\hbar}$$  \hspace{1cm} (1.3)

When the nuclear spin magnetic moment is perpendicular to a static magnetic field it will
experience a torque which will cause the magnetic moment to precess in the plane perpendicular
to the static magnetic field with a frequency given by the Larmor frequency, $\omega_0$. The precession
of the magnetic moment about the static magnetic field causes a time dependent flux in the
radiofrequency coil surrounding the sample, which is then detected as a time dependent current
in that coil. A Fourier transform of this time dependent signal results in a peak at the Larmor
precession frequency $\omega_0$. In the absence of smaller interactions which will be discussed in the
next section the Larmor frequency is given by $\omega_0 = -\gamma B_0$. At a magnetic field strength of
18.8T the Larmor frequencies of $^1$H, $^{13}$C and $^{15}$N are 800.5MHz, 201.3MHz and -81.1MHz
respectively. Due to the importance of the Larmor frequency in any magnetic resonance
experiments Hamiltonians from now on will be written in frequency units with $\hbar$ divided out.

1.4 General NMR Hamiltonian and Interactions

In all NMR experiments the splitting between the two energy levels (and therefore the
Larmor frequency) is altered by a number of interactions that depend on the nuclear structure as
well as the environment surrounding a nucleus which is determined by the local molecular
structure. These interactions include the chemical shift interaction which is caused by a nucleus interacting with the magnetic field of electrons in the sample and J-couplings which is an internuclear interaction mediated by electrons. Also the dipolar coupling and quadrupole interactions are caused by a nucleus interacting with the magnetic moment of other nuclei and with higher order magnetic moments respectively. However the quadrupole interaction is not observed in the current experiments because the only nuclei being observed are $^1$H, $^{13}$C and $^{15}$N which are all spin ½ nuclei and do not have a quadrupolar moment. But in general the total nuclear spin Hamiltonian has the following form.

$$H_{NMR} = H_0 + H_{CS} + H_D + H_J + H_Q + H_{rf}$$  \hspace{1cm} (1.4)

The structure dependent interactions each contain a number of terms which have a complicated dependence on the molecular orientation with respect to the static magnetic field. However the Zeeman interaction is much larger in magnitude than any of the other interactions and therefore the eigenstates of the nuclear spin Hamiltonian are to a very good approximation determined by the eigenstates of Zeeman Hamiltonian. Other terms in the spin Hamiltonian that commute with the Zeeman Hamiltonian (and have the same eigenstates as the Zeeman Hamiltonian) will perturb the eigenvalues of the Hamiltonian in a small but measurable way. In the so called secular approximation, terms that do not commute with the Zeeman Hamiltonian (and have different eigenstates from the Zeeman Hamiltonian) cause insignificantly small corrections to the eigenstates of total nuclear spin Hamiltonian and are neglected.

1.4.1 Chemical Shift

As a result of the interaction of electrons surrounding a nucleus with the external magnetic field a change in the motion of the electrons occurs. The nucleus at the centre of the
electron density experiences an additional induced magnetic field from the motion of the electrons known as the chemical shift. Due to the non-uniform distribution of electrons around a nucleus the chemical shift is partially dependent on the orientation of the electron density in the external magnetic field. It can be shown in general that the chemical shift Hamiltonian has the following form:

\[ H_{CS} = -\gamma \tilde{I} \sigma \tilde{B} \]  \hspace{1cm} (1.5)

where \( \tilde{I} \) and \( \tilde{B} \) are the nuclear spin and external magnetic field vectors and \( \sigma \) is the spatially dependent chemical shift tensor.

\[
\sigma = \begin{pmatrix}
\sigma_{xx} & \sigma_{xy} & \sigma_{xz} \\
\sigma_{yx} & \sigma_{yy} & \sigma_{yz} \\
\sigma_{zx} & \sigma_{zy} & \sigma_{zz}
\end{pmatrix} \]  \hspace{1cm} (1.6)

In the principal axis system (PAS) the chemical shift tensor is diagonal:

\[
\sigma = \begin{pmatrix}
\sigma_{11} & 0 & 0 \\
0 & \sigma_{22} & 0 \\
0 & 0 & \sigma_{33}
\end{pmatrix} \]  \hspace{1cm} (1.7)

where the three principal components define the symmetry of the interaction. The isotropic component is given by the rotationally invariant trace of the tensor.

\[
\sigma_{iso} = \frac{1}{3} (\sigma_{11} + \sigma_{22} + \sigma_{33}) \]  \hspace{1cm} (1.8)

The anisotropic chemical shift (CSA) can be defined as follows by an anisotropy parameter \( (\delta) \) and an asymmetry parameter \( (\eta) \).

\[
\delta = \sigma_{33} - \sigma_{iso} \]  \hspace{1cm} (1.8)
The total CS Hamiltonian has the following form after the secular approximation.

\[
H_{\text{CS}} = H_{\text{CS,iso}} + H_{\text{CS,iso}} = -\gamma B_0 \sigma_{\text{iso}} l_z - \gamma B_0 \delta \frac{1}{2} (3 \cos^2 \theta - 1 - \eta \sin^2 \theta \cos(2\phi)) l_z
\] (1.10)

Here \(\theta\) and \(\phi\) are the angles between the PAS and the static magnetic field defined by the orientation of the crystal in the static magnetic field. In a powder sample there are a large number of randomly oriented crystallites which result in a broad distribution of the observable CSA frequencies (Figure 1.2).

The chemical shift is measured as a part per million (ppm) frequency shift with respect to a standard compound.

1.4.2 Dipolar Coupling

The dipolar coupling results from the magnetic field created by the spin of one nucleus at the position of another nucleus. This interaction is described generally by the following Hamiltonian:

\[
H_D = -\frac{\mu_0}{4\pi} \hbar \sum_{i<J} \gamma_i \gamma_j \frac{3(I_i \cdot \vec{r}_{ij}/r_{ij})(I_j \cdot \vec{r}_{ij}/r_{ij}) - I_i I_j}{(r_{ij})^3}
\] (1.11)

where \(\mu_0\) is the permeability of free space, \(\gamma_i\) is the gyromagnetic ratio of the nucleus \(i\), \(\vec{I}_i\) is the nuclear spin operator of nucleus \(i\), while \(\vec{r}_{ij}\) is the internuclear vector connecting the two interacting spins \(\vec{I}_i\) and \(\vec{I}_j\), and \(r_{ij}\) is the internuclear distance. In the secular approximation the dipolar coupling can be written in two forms that depend on whether the two interacting nuclei are the same type (homonuclear) or different type (heteronuclear). The Hamiltonians for the homonuclear dipolar interactions, \(H_{D,II}\), and heteronuclear dipolar interactions, \(H_{D,IS}\), are
completely anisotropic and depend on the angle $\theta_{ij}$ between the internuclear vector $\vec{r}_{ij}$ and the static magnetic field $\vec{B}$.

\[
H_{D,II} = -\frac{\mu_e}{4\pi} \hbar \sum_{i<j} \frac{\gamma_j^2}{(r_{ij})^3} \left( 3 \cos^2 \theta_{ij} - 1 \right) (3l_{iz}l_{jz} - \vec{I}_i \cdot \vec{I}_j) \tag{1.12}
\]

\[
H_{D,IS} = -\frac{\mu_e}{4\pi} \hbar \sum_{i<j} \frac{\gamma_i \gamma_j}{(r_{ij})^3} \left( 3 \cos^2 \theta_{ij} - 1 \right) l_{iz} S_{jz} \tag{1.13}
\]

1.4.3 J-Coupling

Indirect spin-spin coupling or J-coupling is an internuclear interaction that is mediated by the electrons surrounding the two nuclei. Although J-couplings are much weaker than all other interactions in SSNMR their effects can still be observed. The interaction is given by the following Hamiltonian after the secular approximation.

\[
H_J = \sum_{i<j} 2\pi f_{ij} l_{iz} S_{jz} \tag{1.14}
\]

1.5 Radio Frequency Pulses and the Rotating Reference Frame

Radiofrequency (RF) pulses are applied as a time-dependent current, which generates a time-dependent magnetic field inside a solenoid surrounding the sample. The RF Hamiltonian is written in terms of the strength of the generated magnetic field, $B_1$ and the frequency of the pulse, $\omega_{rf}$.

\[
H_{rf} = \gamma B_1 \cos(\omega_{rf}t) l_x \tag{1.15}
\]

The RF field can be expressed as the sums of two contributions which rotate in opposite directions about the static magnetic field. The contribution which rotates in the same direction as the nuclear magnetic moment will have an interaction with the magnetic moment that is much stronger than the pulse that rotates in the opposite as the magnetic moment. The relative effect
of the pulse which rotates opposite the magnetic moment called the Bloch-Siegert Shift and is proportional to \((B_1/2B_0)^2 \approx 10^{-7}\). In a typical NMR experiment \(B_0\) is three or more orders of magnitude larger than \(B_1\) therefore the Bloch-Siegert shift is neglected. Under this approximation the RF Hamiltonian can further be simplified by writing it in what is called the rotating reference frame defined by a rotation at a frequency of \(\omega_{rf}\) in the direction of the precession of the magnetic moment.

\[
H_{rf, rot} = \frac{1}{2} \gamma B_1 I_x = \omega_1 I_x \quad (1.16)
\]

In the rotating reference frame it can easily be shown that the observed isotropic Larmor frequency, \(\omega_{0, rot}\) will be given by \(\omega_0 - \omega_{rf}\). If a radio frequency pulse is applied exactly on resonance with the Larmor frequency (i.e. \(\omega_{0, rot} = \omega_0 - \omega_{rf} = 0\)) then the total NMR Hamiltonian in the rotating frame is only dependent on the radiofrequency Hamiltonian as well as anisotropic interactions. The effect of the anisotropic interactions is neglected due to the short duration of the pulse.

\[
H_{NMR, rot} = H_{rf, rot} = \omega_1 I_x \quad (1.17)
\]

This is analogous to saying that the effective magnetic field in the rotating reference frame is along the x axis. This will cause a torque on the magnetic moment of the sample and cause it to precess in the y-z plane of the rotating reference frame at a frequency given by \(\omega_1\). If a pulse is applied for a period of time such that \(\omega_1 t = \pi/2\) then the magnetic moment is rotated by 90° in the y-z plane (such as a rotation from the +z direction to the y-direction) and is commonly called a 90° pulse. A pulse applied for twice the time required for a 90° pulse would
rotate the magnetic moment through $180^\circ$ (such as from the $+z$ direction to the $-z$ direction) and is commonly called a $180^\circ$ pulse.

In the situation that pulses are applied slightly off resonance the nuclear spin Hamiltonian is determined only partially by the radio frequency Hamiltonian. In the case that $\omega_0 - \omega_{rf} \ll \omega_1$ the total nuclear spin Hamiltonian is still dominated by the radiofrequency Hamiltonian and all other terms are neglected, which is known as the strong pulse approximation. However if $\omega_0 - \omega_{rf} \gg \omega_1$ the radiofrequency Hamiltonian negligibly contributes to the total nuclear spin Hamiltonian and is therefore neglected which is known as the weak pulse approximation.

Additionally, in NMR experiments the rotating reference rotating at a frequency $\omega_{obs}$ is used for all Larmor frequency observations. For technical reasons the observed Larmor frequency in the rotating reference frame can be more accurately measured than in the static frame because $\omega_{0,rot} = \omega_0 - \omega_{obs} \ll \omega_0$.

1.6 Magic Angle Spinning

In a typical powder sample all possible crystallite orientations are present, which results, in very broad spectral lines due to the orientation dependence of the dipolar coupling and anisotropic chemical shift interaction, obscuring the site-specific resolution. The spatial dependence of both the anisotropic chemical shift and the dipolar coupling can be removed through the use of magic angle spinning (MAS). By spinning the sample at an angle of $54.7^\circ$ with respect to the static magnetic field (the magic angle) orientation-dependent interactions are averaged out as long as the spinning frequency is comparable to or larger than the frequency of the interaction. This is illustrated in Figure 1.2 where the broad static solid powder spectra resulting from the chemical shift anisotropy and the dipolar coupling are first split into a number
of spinning sideband peaks, which occur at multiples of the spinning frequency when the sample
is spun slowly about the magic angle. As the spinning rate is increased the intensity of the side
bands decreases and a single strong centerband is observed at the frequency of the isotropic
chemical shift.
Figure 1.2 Simulated spectra of backbone amide $^{15}$N ($\sigma_{iso}=119.6$ ppm) resulting from the N-H dipolar coupling under the static condition and at MAS spinning frequencies of 1 kHz, 4 kHz and 12.5 kHz. Simulation was done with the SPINEVOLUTION software$^{30}$ using the dipolar coupling constant $b_{IS}=11.5$ kHz corresponding to the backbone N-H internuclear distance of 1.01 Å.$^{32}$
1.7 Multidimensional Spectroscopy

A one dimensional spectrum will contain at least one peak for each site in the protein occupied by the observed nuclei. For a large protein like ASR there will too many peaks in $^{15}\text{N}$ or $^{13}\text{C}$ spectrum with most peaks overlapped eliminating site-specific resolution. In this case site-specific resolution is achieved by spreading the observed peaks into multiple dimensions by performing multidimensional correlation experiments. In a typical two-dimensional $^{15}\text{N}$-$^{13}\text{C}_\alpha$ experiment the magnetization is initially transferred to the backbone from $^1\text{H}$ amide nucleus $^{15}\text{N}$, where the magnetization evolves under isotropic chemical shift of that nucleus for time $t_1$, then the magnetization is transferred to the backbone $^{13}\text{C}_\alpha$ nucleus where the signal evolution is detected. This process is repeated for a range of values for $t_1$ (212 points in the range of 0 ms-15 ms is typical) resulting in a two-dimensional representation of the NMR signal that included both the chemical shift of the amide $^{15}\text{N}$ and the backbone $^{13}\text{C}_\alpha$. A 2D Fourier transform of this signal results in a 2D spectrum with a peak for every $^{15}\text{N}$-$^{13}\text{C}_\alpha$ spin pair in the protein, meaning that one peak will be observed for every residue and since these peaks are spread out in two dimensions this allows the site-specific resolution of many residues in the protein.

In SSNMR magnetization is transferred from one nucleus to a subsequent nucleus by using the through-space dipolar interaction. For heteronuclear signal transfer in proteins a spinlock field is applied to each nucleus at a strength $\omega_{1,i}$. Signal is transferred between the two nuclei, $I$ and $S$, if the sum or difference between $\omega_{1,I}$ and $\omega_{1,S}$ is equal to $n \cdot \omega_r$, where $n=1$ or $n=2$. This is commonly called the Hartmann-Hahn (HH) matching condition. Other techniques are available for both homonuclear and heteronuclear signal transfer but are not relevant to the present work.
Nearly complete resolution of all sites in the protein can be obtained by performing three-dimensional correlation experiments. Where the magnetization is transferred through three nuclei such that it is able to evolve under chemical shift interaction of each nucleus. However the experimental time increases substantially with each dimension from minutes for a one-dimensional experiment to days for a three-dimensional experiment.

1.8 NMR Signal Relaxation

During an NMR experiment the magnetic moment of the sample undergoes a precession in the plane perpendicular to the static magnetic field. However the observed precession does not occur indefinitely. The magnitude of the magnetic moment exponentially decreases in the transverse plane while simultaneously the thermal equilibrium state of the magnetic moment aligned with the static magnetic field is exponentially recovered. These exponential processes are characterized by two time constants, the longitudinal relaxation time $T_1$ and the transverse relaxation time $T_2$. The longitudinal relaxation time $T_1$ characterizes the time required for the system to return to thermal equilibrium and the transverse relaxation time $T_2$ characterizes the time for the magnetic moment to decay in the transverse plane.

These time constants are fundamentally related to thermal motions of nuclei within the sample that cause fluctuations in the local magnetic field due to the orientation dependence of the CSA and dipolar interactions. The fluctuations in the local magnetic field induce transitions between the nuclear spin eigenstates, which overall causes the system to return to equilibrium. In the simplest model local motions are assumed to be completely random which results in a single exponential decay for the auto correlation function of the local magnetic field. The Fourier transform of the autocorrelation function, called the spectral density $J(\omega)$, gives the frequency distribution of the local fluctuations.
\[ J(\omega) = 2\langle B_x^2 \rangle \frac{\tau_c}{1 + \omega^2 \tau_c^2} \]  

(1.18)

Here \( \langle B_x^2 \rangle \) is the amplitude of the local field fluctuations and \( \tau_c \) is the correlation time which is characteristic of the overall time scale of the motion. The contributions of the CSA and dipolar interactions to the relaxation rate constants \( R_1 \) and \( R_2 \), which are the inverse of the relaxation times \( T_1 \) and \( T_2 \), have the following relations to the spectral density function solved at transition frequencies in MAS SSNMR.\(^{15,34}\)

\[ R_{1\text{CSA}} = \frac{3}{4} (\delta \omega_I)^2 J(\omega_I) \]  

(1.19)

\[ R_{1\text{IS}} = \frac{b_{1S}^2}{4} \left( J(\omega_I - \omega_S) + 3J(\omega_I) + 6J(\omega_I + \omega_S) \right) \]  

(1.20)

\[ R_{2\text{CSA}} = \frac{(\delta \omega_I)^2}{8} \left( 4J(0) + 3J(\omega_I) \right) \]  

(1.21)

\[ R_{2\text{IS}} = \frac{b_{1S}^2}{8} \left( 4J(0) + J(\omega_I - \omega_S) + 3J(\omega_S) + 6J(\omega_I) + 6J(2\omega_I) \right) \]  

(1.22)

Here \( \delta \) is the chemical shift anisotropy (97.67 ppm for backbone amide \(^{15}\)N)\(^{35,31}\) and \( b_{1S} \) is the dipolar coupling constant (7.43\times10^4 \text{ rad/s for } ^{15}\text{N}-^1\text{H}).\(^{36}\) The total relaxation rates are the sum of the relaxation rates due to the CSA and dipolar interactions.

\[ R_1 = \frac{1}{T_1} = R_{1\text{CSA}} + R_{1\text{IS}} \]  

(1.23)

\[ R_2 = \frac{1}{T_2} = R_{2\text{CSA}} + R_{2\text{IS}} \]  

(1.24)

Due to the direct relation of NMR signal relaxation to the spectral density of motions measurement of the \( T_1 \) and \( T_2 \) relaxation time constants can in principle fully quantify the amplitude and time scale of motions present in the sample. However, the auto correlation function of molecular motions is more complicated than the single exponential decay used here.
Full expressions for the autocorrelation function and the spectral density in the general case can be found in reference 37.

1.9 Dipolar Chemical Shift Spectroscopy

Valuable information about the sample can be obtained by measuring the site-specific strength of the dipolar coupling between two nuclei using Dipolar Chemical Shift (DIPSHIFT) Spectroscopy. Under MAS conditions, used to average out anisotropic interaction and obtain high spectral resolution, dipolar recoupling pulse sequences are applied in an additional spectral dimension to site-specifically measure dipolar interactions. A number of dipolar recoupling pulse sequences have been developed for use in DIPSHIFT experiments on biomolecules. In this study the T-MREV (transverse-Mansfield, Rhim, Elleman and Vaughan) sequence for recoupling of $^{15}\text{N}-^{1}\text{H}, ^{13}\text{C}-^{1}\text{H}$ couplings and the ZF-TEDOR (Z-Filtered Transferred Echo Double Resonance) recoupling sequence for $^{15}\text{N}-^{13}\text{C}$ dipolar couplings are used. The strength of the dipolar coupling given by the dipolar coupling strength ($b_{IS}$) is obtained by site-specifically following the evolution of the signal under dipolar recoupling and fitting the signal to a theoretically predicted signal evolution for the specific recoupling sequence. The observed dipolar coupling strength is used here to determine the mobility of the two nuclei. But in previous studies similar experiments have been used to determine the distance between the two nuclei or the backbone torsion angles.

The experimentally observed dipolar coupling strength between two nuclei that are subjected to motions is partially reduced due to motional averaging caused by the reorientation of the internuclear vector. The extent of motional averaging depends on the amplitude of motions and is quantified by an order parameter $S$ given by the ratio of the experimentally observed dipolar coupling constant and its rigid limit.
Often the motion of the internuclear vector is modelled as the free isotropic diffusion of
the vector within a cone. In this model the order parameter can be directly related to the cone
angle that defines the magnitude of the free diffusion of the internuclear vector.\textsuperscript{10,11}

\[ S = \frac{b_{15,\text{obs}}}{b_{15,\text{rigid}}} \]  \hspace{1cm} (1.25)

\[ S = \frac{1}{2}\cos \theta (1 + \cos \theta) \]  \hspace{1cm} (1.26)

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47.


Chapter 2 Experimental Details and Theory

2.1 Sample Preparation

Three ASR samples were used in these studies. All DIPSHIFT experiments were conducted on two ASR samples with uniform $^{15}$N labeling but alternative $^{13}$C labeling, grown on glycerol labeled at positions 1 and 3 (1,3-ASR in the following) or at position 2 (2-ASR). Measurements of $R_{1p}$ were carried out on a uniformly $^{13}$C, $^{15}$N-labeled ASR (UCN ASR in the following). Samples were prepared as described previously. Briefly, C-terminally truncated histidine-tagged ASR was expressed in BL21-Codonplus-RIL E. coli grown on M9 minimal medium using 1 g of $^{15}$N-labelled ammonium chloride as the sole nitrogen source, and 4 g of either 2-$^{13}$C or 1,3-$^{13}$C labeled glycerol or $^{13}$C$_6$-labeled glucose as carbon sources for alternatively or uniformly labeled samples, respectively. Retinal was added exogenously at the time of induction at a concentration of 7.5 $\mu$M.

The cells were collected by centrifugation and then treated with lysozyme and DNase I before breaking by sonication. The membrane fraction was solubilized in 1% DDM (n-dodecyl $\beta$-D-maltoside) at 4 °C. Liposomes were prepared by hydrating dried DMPC and DMPA added in 9:1 ratio (w/w). Liposomes were mixed with purified ASR solubilized in detergent, at a protein:l lipid ratio of 2:1 (w/w). Bio-beads were used for detergent removal. Proteoliposomes were collected by ultracentrifugation at 900,000 g for 6 h, and packed into thin wall 3.2 mm rotors for DIPSHIFT experiments. The UCN ASR sample was packed into a 1.3 mm NMR rotor for $R_{1p}$ measurements.
2.2 SSNMR spectroscopy

Dipolar order parameters for four couplings were measured using 3D dipolar chemical shift correlation (DIPSHIFT) experiments, which employ 2D NCA or NCO chemical shift correlation spectroscopy, and Z-filtered TEDOR (ZF TEDOR)\(^3,4\) for \(^{15}\text{N}-^{13}\text{C}_\alpha\) and \(^{15}\text{N}-^{13}\text{C}'\) coupling measurements, or the Transverse MREV (TMREV)\(^5\) recoupling to probe the strengths of \(^{15}\text{N}-^1\text{H}\) and \(^{13}\text{C}-^1\text{H}\) interactions. Details of pulse sequences are given in Figure 2.1. Site-specific measurements of \(^{13}\text{C}_\alpha-^1\text{H}_\alpha\) couplings were conducted using 2D NCA spectroscopy on 1,3-ASR and 2-ASR samples, cumulatively allowing the resolution of 68 \(^{13}\text{C}_\alpha-^1\text{H}_\alpha\) couplings in total. The measurements of \(^{15}\text{N}-^1\text{H}\) coupling were conducted on both 1,3-ASR and 2-ASR samples. Residue specificity was achieved using NCA spectra, with “afterglow” magnetization used to record complementary NCO spectra. A total of 92 \(^{15}\text{N}-^1\text{H}\) couplings were determined in these experiments.\(^6\) Measurements of couplings were conducted on the 1,3-ASR and 2-ASR samples by using ZF TEDOR to simultaneously transfer polarization from \(^{15}\text{N}\) to \(^{13}\text{C}_\alpha\) and \(^{13}\text{C}'\) and record complementary NCA and NCO spectra. A total of 75 \(^{15}\text{N}-^{13}\text{C}_\alpha\) and 50 \(^{15}\text{N}-^{13}\text{C}'\) couplings were determined in these experiments.

All dipolar chemical shift (DIPSHIFT) correlation experiments were performed on a Bruker Avance III 600 MHz spectrometer, using 3.2 mm triple resonance (HCN) E-free probe for ZF TEDOR measurements, or 3.2 mm HCN TL2 probe for TMREV experiments. Sample temperature was 280 K in all experiments. 84 kHz SPINAL-64 decoupling\(^7\) was used during both the direct and indirect chemical shift evolution periods.
Figure 2.1 N-H T-MREV heteronuclear dipolar recoupling pulse sequence for measuring backbone $^{15}$N-$^1$H dipolar coupling (A) and $^{13}$C-$^1$H dipolar coupling (B). N-Cα correlation spectra and afterglow N-C' correlation spectra (for $^{15}$N-$^1$H only) are recorded as a function of TMREV mixing time ($t_{\text{mix}}$). The TMREV pulse train is rotor synchronized with $n=4$ pulse sequences applied per rotor period and with each pulse sequence cyclically phased over one rotor period. The TMREV pulse train is applied an integer number of times for the duration of $t_{\text{mix}}$. Following the TMREV recoupling period TPPM decoupling is applied until the completion of $m$ rotor periods after the start of the TMREV recoupling sequence (in our experiments $m=12$). Afterglow N-C' spectra are collected by storing the residual magnetization on $^{15}$N following N-Cα cross polarization (CP) along the z-axis during the first acquisition. After the first acquisition is complete the *-stored magnetization is then transferred to C' for the second acquisition. White boxes represent 90° pulses, black boxes represent 180° pulses. In (A) pulses are phase cycled by $\phi_1=y-y, \phi_2=x, \phi_3=x, \phi_4=x y y x y -x -x -y -y, \phi_5=x y y x y -x -x -y -y, \phi_6=y, \phi_7=x y y x y -x -x -y -y, \phi_8=y, \phi_{\text{rec}}=x -x -y -x -y, \phi_{\text{rec}}=x -x y -x -y -y, \phi_{\text{rec}}=x x y -x -y -y$. All pulse phases not indicated are given with phase x.
The TMREV DIPSHIFT experiments were performed at a spinning frequency of 8 kHz. They start with $^1$H/$^{15}$N cross polarization (CP)$^8$ of 2 ms duration with the $^{15}$N field strength of 35 kHz, and the proton field ramped by 10% around the n=2 Hartmann-Hahn (HH)$^9$ matching condition. $^{15}$N/$^{13}$C$_\alpha$ band-selective CP$^{10}$ was done using a 5 ms contact time with a 22 kHz spinlock field on $^{15}$N, and with the carbon field ramped linearly (10%) around the n=1 HH condition. TMREV measurements of N-H order parameter were implemented with the “afterglow” magnetization detection step following Banigan et al.$^6$ The magnetization remaining on the $^{15}$N after the $^{15}$N/$^{13}$C$_\alpha$ CP was stored along the z-axis and used to record an additional NCO correlation spectrum (Figure 2.1), the afterglow $^{15}$N/$^{13}$C$'$ band-selective CP was done using the same parameters for $^{15}$N/$^{13}$C$_\alpha$ except that the $^{13}$C carrier channel was placed at 175 ppm in the middle of the carbonyl band.

T-MREV recoupling was implemented in a constant time manner as shown in Figure 2.1, with four T-MREV elements per rotor cycle (TMREV-4), with the proton radio frequency (RF) field strength of ~96 kHz (90° pulse duration of 2.6 μs). The total echo period was set to 12 rotor cycles. TPPM decoupling used during the echo period was performed at 96 kHz.

ZF-TEDOR DIPSHIFT experiments were performed at a spinning frequency of 12 kHz, using an “out and back” scheme$^4$ (see Figure 2.2). The initial $^1$H/$^{13}$C CP was performed with a 2 ms contact time, the $^{13}$C RF field of 50 kHz, and with a proton field linearly ramped around n=1 HH condition (10% ramp). Polarization transfer between $^{13}$C and $^{15}$N was accomplished using the TEDOR method with REDOR pulse trains implemented on the nitrogen channel, with $^{15}$N 180° pulses of 14 μs. TPPM proton decoupling$^{11}$ of 90 kHz was used during REDOR$^{12}$ periods. $^{13}$C 90° and 180° pulses were 4 μs and 8 μs, respectively. Z-filters of 167 μs duration
(two rotor cycles) with the RF proton field of 12 kHz were used after both REDOR pulse trains to remove artifacts from the remaining homonuclear J-couplings between carbon spins.\(^4\)

\begin{figure}
\centering
\includegraphics[width=\textwidth]{ZF-TEDOR_sequence}
\caption{ZF-TEDOR dipolar recoupling pulse sequence for simultaneous measurement of \(^{15}\)N-\(^{13}\)C\(\alpha\) and \(^{15}\)N-\(^{13}\)C' dipolar coupling by observing N-C\(\alpha\) and N-C' spectra as a function of dipolar recoupling time \((t_{\text{mix}})\). Dipolar recoupling with the REDOR pulse sequence is used to transfer magnetization to \(^{15}\)N using the “out and back” scheme. The REDOR pulse sequence is rotor synchronized with two pulses every rotor period and phase cycled using \(xy\)-4 to compensate for RF mismatch. The delay \(\kappa\) is calculated such that the time between the first and second REDOR periods is equal to an integer number of rotor cycles. White boxes represent 90° pulses, black boxes represent 180° pulses. The phases of all pulses are given as \(\phi_1 = 16x(x)\), \(\phi_2 = x\), \(\phi_3 = x\), \(\phi_4 = x\), \(\phi_5 = y\), \(\phi_6 = x\), \(\phi_{\text{rec}} = y\). Unless otherwise stated all remaining pulses are of phase x.}
\end{figure}

Measurements of \(^{15}\)N transverse \(R_{1p}\) relaxation constants were performed on a Bruker 800 MHz Avance III spectrometer using 1.3 mm ultrafast MAS Bruker probe tuned to \(^1\)H, \(^{13}\)C, and \(^{15}\)N. A spinning frequency of 50 kHz was used in all measurements, with the sample temperature kept at 280 K. \(^1\)H/\(^{15}\)N cross polarization was performed with a contact time of 2 ms, 30 kHz RF field on \(^{15}\)N, and a 10% ramp around 80 kHz on \(^1\)H. \(^{15}\)N/\(^{13}\)C\(\alpha\) band selective CP was implemented using a 5 ms contact time matching the \(n=1\) double-quantum HH matching condition using an RF field of 20 kHz on nitrogen, and a 10% ramped RF field centered at 30 kHz on \(^{13}\)C. No proton decoupling was used during an \(^{15}\)N/\(^{13}\)C\(\alpha\) CP. The \(^{15}\)N transverse magnetization decay was probed at \(^{15}\)N spin lock fields of 10 kHz, 12 kHz, 14 kHz and 16 kHz.
For each of the field strengths, five points were taken for the duration of the spin lock of 0 ms, 20 ms, 50 ms, 100 ms and 200 ms (Figure 2.3). Spin lock power was calibrated at 50 kHz using the Rotary Resonance recoupling, by following the $^{15}$N signal intensity as a function of lock field.$^{13,14}$ Low power decoupling was used during both direct and indirect acquisition with a proton RF field optimized around 12 kHz. Recycle delay of 600 ms was used in these experiments.

**Figure 2.3** Pulse sequence for measurement of relaxation time in the rotating reference frame ($T_{1\rho}$). N-CA correlation spectra are recorded as a function of a spinlock relaxation time ($\tau$). White boxes represent 90° pulses, black boxes represent 180° pulses. The phases of all pulses are given as $\phi_1$=y -y, $\phi_2$=x, $\phi_3$=x, $\phi_4$=x, $\phi_5$=x y y -x -x -y -y, $\phi_{rec}$=x -x y -x -y x -y y. The phase of all other pulses including the spinlock pulse is x.

### 2.3 Data Analysis

Carbon chemical shifts were indirectly referenced to 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) by adjusting the position of the $^{13}$C adamantane down field peak to 40.48 ppm.$^{16}$ The nitrogen chemical shifts were referenced indirectly to $^{13}$C chemical shifts. T-MREV and ZF-TEDOR spectra were processed by NMRPipe$^{17}$ using Lorentzian-to Gaussian window function with line broadening of 60 Hz and line narrowing of 30 Hz in the direct dimension with
30 Hz line broadening and 12 Hz of line narrowing in the indirect dimension. Spectra from R\textsubscript{1ρ} experiments were processed twice by NMRPipe\textsuperscript{17} using Lorentzian-to-Gaussian-window function with two separate sets of parameters to obtain two separate representations of each spectrum. The first processing used line narrowing of 80 Hz and line broadening of 40 Hz in the direct dimension with 90 Hz line narrowing and 40 Hz of line broadening in the indirect dimension to increase signal to noise ratio of isolated cross-peaks. The second processing used line narrowing of 70 Hz and line broadening of 40 Hz in both the direct and indirect dimension to increase resolution of the overlapped peaks. Peak amplitudes from all experiments were extracted using the CARA software\textsuperscript{18} based on previously reported assignments shown in Figure 1.1 (BMRB entry 18595).\textsuperscript{19} Within CARA the amplitudes of completely isolated peaks were taken without modification, while partially overlapped peaks were fit to Gaussian line shapes, and linear deconvolutions were performed with neighbouring peaks to estimate their amplitudes. The extracted peak amplitudes were fit to theory (as described below) to determine the one bond order parameters \langle S \rangle and the relaxation rates in the rotating reference frame R\textsubscript{1ρ}.

2.3.1 Determination of 15N-1H and 13C-1H dipolar order parameters by T-MREV

Amplitudes from T-MREV experiments were fit using the theory by Hohwy et al.\textsuperscript{5} which is outlined here. The first order average Hamiltonian for two interacting spins I and S is given here in terms of the spin operators \( I_+ \), \( I_- \) and \( S_z \).

\[
H^{(1)}_{IS} = \kappa \omega^{(-1)} I_+ S_z + \kappa^* \omega^{(1)} I_- S_z
\]  

(2.1)

Here \( \kappa \) is the complex scaling factor determined theoretically by the number of T-MREV pulse sequences per rotor cycle, \( n \) (\( \kappa =0.485 \) for \( n=4 \)). Also the dipolar coupling frequency \( \omega \) is given in terms of the Euler angles (\( \beta \) and \( \gamma \)) and the dipolar coupling constant \( D_{IS} \).

\[
\omega^{(1)} = (\omega^{(-1)})^* = \frac{h \tau S_z}{2 \sqrt{2}} \sin(2\beta) e^{i\gamma} 
\]  

(2.2)
During the T-MREV pulse sequence a nucleus $S$ which is initially in the state $S_x$ interacts with one other nucleus $I$ causing the state to evolve into $S_x$ and $S_y I_{\psi}$ states. In the protein backbone the relevant interactions are between the nuclei $^{15}\text{N}/^{13}\text{C}$ and their nearest neighbour $^1\text{H}$ as well as a distant $^1\text{H}$. In this case the signal will evolve into four states $S_x, S_y I^1_y, S_y I^2_y$ and $S_y I^1_y I^2_y$. The signal evolution is given by the following differential equation.

$$\begin{pmatrix}
\frac{d}{dt} \langle S_{jx} \rangle \\
\langle S_{jy} I_{1\psi} \rangle \\
\langle S_{jy} I_{2\psi} \rangle \\
\langle S_{jy} I_{1\psi} I_{2\psi} \rangle
\end{pmatrix}
= \begin{pmatrix}
-R_{2,1} & -\omega_{1j} & -\omega_{2j} & 0 \\
\omega_{1j} & -R_{2,2} & 0 & -\omega_{2j} \\
\omega_{2j} & 0 & -R_{2,2} & -\omega_{1j} \\
0 & \omega_{2j} & \omega_{1j} & -R_{2,3}
\end{pmatrix}
\begin{pmatrix}
\langle S_{jx} \rangle \\
\langle S_{jy} I_{1\psi} \rangle \\
\langle S_{jy} I_{2\psi} \rangle \\
\langle S_{jy} I_{1\psi} I_{2\psi} \rangle
\end{pmatrix} \tag{2.3}
$$

Where $R_{2,i}$ are the relaxation rates for states involving coherences with $i-I$ $^1\text{H}$ and $\omega_{ij}$ is the magnitude of the effective coupling frequency between nuclei $S_j$ and $I_i$. The signal evolution was calculated by numerically diagonalizing this matrix using standard algorithms in the GNU Scientific Library and implemented via an in-house C program. The parameters in the equation were calculated as follows:

The relaxation rate $R_{2,1}$ was set to zero due to the constant time implementation of the T-MREV pulse sequence while the other relaxation times are related to the transverse signal relaxation rate $R_2$ which was a fit parameter.²

$$R_{2,2} = \frac{2}{3} R_2 \tag{2.4}$$

$$R_{2,3} = \frac{9}{8} R_2 \tag{2.5}$$

Also the magnitude of the effective coupling frequency between nuclei $S_j$ and $I_i$ was calculated as follows.

$$\omega_{ij} = \left| \omega_{I_{1}S_{j}}^{(1)} \right| = \left| \omega_{I_{1}S_{j}}^{(-1)} \right| = \kappa \frac{b_{II} \epsilon_{f}}{2v^2} \sin(2\rho_{ij}^{CR}) \tag{2.6}$$
Here $\kappa$ is the magnitude of the complex scaling factor determined theoretically by the number of T-MREV pulse sequences per rotor cycle, $n$ ($\kappa = 0.485$ for $n=4$), $\beta_{ij}^{CR}$ is the Euler angle relating the internuclear vector in the crystal frame to the rotor frame and $b_{I_s J}$ is the dipolar coupling constant for the nuclei $S_j$ and $I_i$. For each simulation a powder average over $\beta_{ij}^{CR}$ was performed. The dipolar coupling constant has the following standard form.

$$b_{I_s J} = -\frac{\mu_0}{4\pi} \hbar \frac{\gamma_J}{(r_{ij})^3}$$  \hspace{1cm} (2.7)

The dipolar coupling between the nucleus $S_j$ and its nearest neighbour $^1\text{H}$, $b_{I_s J}$ was the second fit parameter. The dipolar coupling constant between nucleus $S_j$ and its next nearest neighbour $^1\text{H}$, $b_{I_{s+1} J}$, was calculated based on the inter nuclear distances of 2.16\AA\ between $^{13}\text{C}_\alpha$ and $^1\text{H}_N$ or 2.09\AA\ between $^{15}\text{N}$ and $^1\text{H}_C\alpha$ based on the geometry in Reinstra et. Al.\(^2\).

Experimental data was fit to the numerically calculated simulations using the already mentioned fit parameters $R_2$ and $b_{I_s J}$ as well as an overall amplitude scaling factor. The best fit dipolar coupling constant between the nucleus $S_j$ and its nearest neighbour $^1\text{H}$ is compared to the dipolar coupling constant in the rigid limit using the known bond lengths. In the rigid limit the nearest neighbour $^1\text{H}$ was considered to be at a distance of 1.01 \AA\ relative to $^{15}\text{N}$ and 1.10 \AA\ relative to $^{13}\text{C}$ based on neutron diffraction experiments\(^3\). The dipolar order parameter was determined as ratios $< S > = b_{IS,\text{expt}}/b_{IS,\text{rigid}}$ between the experimentally determined dipolar coupling, $b_{IS,\text{expt}}$ and its rigid limit $b_{IS,\text{rigid}}$.

### 2.3.2 Determination of 15N-13Cα and 15N-13C’ dipolar order parameters by ZF-TEDOR

Amplitudes from ZF-TEDOR experiments were fit to theory presented in Jaroniec et. al.\(^4\) and briefly outlined here. The magnetization transferred back to the $^{13}\text{C}$ nucleus ($V$) is the sum of
the magnetization that comes from 2 interacting $^{15}$N nuclei ($V_1$ and $V_2$). The signal that results from the $^{13}$C nucleus interacting with the the two $^{15}$N nuclei is expressed as a function of the TEDOR mixing time ($t_{mix}$).

$$V_1(t_{mix}) = \Lambda \left(1 - \left[J_0(\sqrt{2} \frac{b_{115}}{2\pi} t_{mix})\right]^2\right) \left(1 + \left[J_0(\sqrt{2} \frac{D_{12}}{2\pi} t_{mix})\right]^2\right)$$

$$V_2(t_{mix}) = \Lambda \left(1 - \left[J_0(\sqrt{2} \frac{b_{115}}{2\pi} t_{mix})\right]^2\right) \left(1 + \left[J_0(\sqrt{2} \frac{D_{12}}{2\pi} t_{mix})\right]^2\right)$$

Here $J_0(x)$ is the zeroth order Bessel function, $b_{115}$ is the dipolar coupling constant between the $^{13}$C nucleus and the $ith$ $^{15}$N nucleus and $\Lambda$ is given by

$$\Lambda = \frac{1}{2} \lambda \exp(-Rt_{mix})$$

where $Rt_{mix}$ is the signal relaxation rate and $\lambda$ is an overall amplitude scaling factor. Also the effects of Cα-C’ J-couplings are neglected due to the selective isotopic labeling system used, which in almost all cases results in $^{13}$C labelling of Cα or C’ but not both. The rigid limit dipolar coupling constants of the nearest neighbour $^{15}$N were calculated using the standard one-bond lengths of 1.46 Å and 1.33 Å for N-Cα and N-C’ bonds, respectively. The distant $^{15}$N was placed at a distance of 2.41 Å for Cα and 2.45 Å for C’ as determined by the geometry in Rienstra et. al.\(^1\)

The experimental signal was fit to the ZF-TEDOR signal evolution simulations where the fit parameters included were overall amplitude, relaxation time and the one bond N-C dipolar coupling constant and dipolar order parameter.

2.3.3 Determination of Motional Correlation Time, $\tau$, by Measurement of the Relaxation Rate in the Rotating Reference Frame, R1ρ

The time scale of motions was determined by measurement of the $^{15}$N transverse relaxation rates. The peak amplitudes from all relaxation experiments were fit to a single
exponential by fitting the rate of signal decay during the spin lock ($R_{1\rho}$) and an overall amplitude factor.

The NMR spin-lattice relaxation $R_2$ or the spin-lattice relaxation in the rotating frame, $R_{1\rho}$, are sensitive reporters on motions occurring on a slow, nanosecond-millisecond time scale. Although in the solid-state these relaxation rates are typically dominated by coherent contributions related to incomplete averaging of the heteronuclear and homonuclear dipolar interactions by the magic angle spinning and proton decoupling, Emsley and co-workers have recently demonstrated that these contributions to the $R_{1\rho}$ relaxation rates are effectively suppressed under fast MAS (spinning frequencies greater than 45 kHz) and with the $^{15}$N spinlock power greater than 10 kHz (and away from rotary recoupling conditions$^{13,14}$). Under these conditions, $R_{1\rho}$ rates are dominated by the stochastic contributions, and could yield information on the molecular motions in microcrystalline GB1.$^{22}$

Spin-lattice $^{15}$N $R_{1\rho}$ rotating frame relaxation rate measurements were conducted at a spinning frequency of 50 kHz using 1.3 mm MAS probe. Because of the reduced amount of sample in a 1.3mm rotor, large molecular weight of the protein, and the presence of lipids, site specific relaxation measurements are impeded by the low sensitivity and are time consuming. Therefore measurements were conducted on a single uniformly $^{13}$C,$^{15}$N ASR sample. To confirm that fast MAS suppresses coherent contributions resulting in significantly smaller relaxation rates in membrane proteins in a lipid environment, the bulk $R_{1\rho}$ as a function of lock field was initially estimated, as shown in Figure 2.4. In a qualitative agreement with the results obtained on GB1, a significant decrease in relaxation rates with increasing spin lock field was observed, with $T_{1\rho}$ reaching a plateau at $\sim 2.8$ s$^{-1}$ ($T_{1\rho} \sim 360$ ms) at spinlock fields higher than $\sim 10$ kHz.$^{22}$
Figure 2.4 Bulk amide $^{15}$N $R_{1\rho}$ as a function of spinlock power level. Data were recorded at 800 MHz, and at a spinning frequency 50 kHz. The data are in a qualitative agreement with Lewandowski et Al.\textsuperscript{22}

More definitive conclusions on the time scale of motions experienced by various residues in ASR could be derived for the theoretical model developed by Kurbanov et al.\textsuperscript{23} This model provides estimates for $R_{1\rho}$ relaxation rates for a spin S subjected to chemical shift anisotropy and coupled through-space to another spin in the presence of MAS:

$$R_{1\rho} = \frac{1}{2} R_{1}^{CSA} + \frac{1}{2} R_{1}^{IS} + R_{1\Delta}^{CSA} + R_{1\Delta}^{IS}$$  \hspace{1cm} (2.10)

where $R_{1}^{CSA}$ and $R_{1}^{IS}$ are the longitudinal relaxation rates resulting from the anisotropic chemical shift and dipolar coupling while $R_{1\Delta}^{CSA}$ and $R_{1\Delta}^{IS}$ are the additional dependence on the transverse relaxation rate. These relaxation rates have the following form.

$$R_{1}^{CSA} = \frac{3}{4} (\delta \omega_1)^2 J(\omega_1)$$  \hspace{1cm} (2.11)

$$R_{1}^{IS} = \left(\frac{b_{IS}}{2}\right)^2 \left(J(\omega_1 - \omega_S) + J(\omega_1) + J(\omega_1 + \omega_S)\right)$$  \hspace{1cm} (2.12)

$$R_{1\Delta}^{CSA} = \frac{1}{6} (\delta \omega_1)^2 \left\{ \frac{1}{2} J(\omega_1 - 2 \omega_R) + J(\omega_1 - \omega_R) \right\}$$  \hspace{1cm} (2.13)
\[ R_{1\Delta}^{1S} = \frac{1}{6} (b_{IS}/2)^2 \left\{ 3f(\omega_S) + \frac{1}{3} f(\omega_1 - 2\omega_R) + \frac{2}{3} f(\omega_1 - \omega_R) \right\} + \frac{2}{3} f(\omega_1 + \omega_R) + \frac{1}{3} f(\omega_1 + 2\omega_R) \]  

(2.14)

where \( \delta \omega_I \) is the strength of the anisotropic chemical shift, \( \eta \) is the CSA asymmetry parameter, \( b_{IS} \) is the strength of the dipolar coupling and \( J(\omega) \) or \( J(\eta,\omega) \) is the spectral density function (in the limit where \( \eta=0, J(\eta,\omega)=J(\omega) \)). Here, the spectral density function \( J(\omega) \) is related to motional parameters, such as the correlation time \( \tau_c \), and the order parameter describing the amplitude of motion of the N-H bond vector, \( S \):

\[ J(\omega) = \frac{2}{5} (1 - S^2) \frac{\tau_c}{1 + \omega^2 \tau_c^2} \]  

(2.15)

\( \omega_\rho \) is the effective field strength during the spinlock period expressed in rad/sec (in this case close to the RF power, \( \omega_1 \)), \( \omega_R / 2\pi \) is the spinning frequency, while \( \omega_I \) and \( \omega_S \) are the Larmor frequencies of the two nuclei (here \(^1\text{H}\) and \(^{15}\text{N}\)).

### 2.4 Error Analysis

In all experiments, statistical analysis of random errors was performed using in-house Monte Carlo simulations, implemented using the C programing language. Briefly, the experimental data is fit to the corresponding theory to determine a best fit simulation. Gaussian random noise with a distribution determined by the rms spectral noise was then added to best fit simulation of experimental data to produce a set of 5000 experimental simulations with Gaussian random noise. The set is then refit to theory to determine a distribution of fit parameters based on the distribution of random noise.

Systematic errors originating from pulse imperfections and RF inhomogeneity, etc., were estimated using Spinevolution.\(^{24}\) In T-MREV experiments it was found that a \(^1\text{H}\) RF power level
error of 5%, resulting from RF inhomogeneity, $^1$H spectral width or calibration uncertainty, would result in a 3% error in the observed order parameter. In ZF-TEDOR experiments a $^{15}$N RF power level error of 5% would only result in a 0.6% error in the observed order parameter.

References


Chapter 3 Results

3.1 Probing dipolar order parameters

The 3D CONCA experiment establishes correlations between the backbone atoms, $^{13}$C', $^{15}$N, $^{13}$C$_\alpha$, provides nearly complete resolution of the backbone sites (Figure 3.1A), and allows detailed site-specific investigation of the polarization transfer efficiencies. In Figure 3.1B the plot of cross peaks intensities as a function of residue is shown, which serves as a preliminary indicator of the local protein mobility. While most residues can be detected in this spectrum, the cross peak intensities indicate a clear and expected trend: the transmembrane regions appear to be generally more rigid, while cross peak intensities decrease towards the solvent exposed flanks of helices and in the loops. One notable exception is the B-C loop on the extracellular side, which appears to be less mobile. Indeed, the B-C loop is well structured and forms a short but well-defined $\beta$-hairpin in the SSNMR structure of ASR.$^1$

Figure 3.1 Three-dimensional CONCA spectroscopy. (A) Representative F$_2$-F$_3$ (NCA) 2D plane of a 3D CONCA experiment. Cross peaks are labeled according to the $^{15}$N/$^{13}$C$_\alpha$ assignments. (B) Signal to noise ratios of individual cross peaks detected in a 3D CONCA experiment on the UCN ASR plotted as a function of residue number. The spectrum was recorded at a proton frequency of 800 MHz as discussed previously.$^2$ Secondary structure of ASR derived from the chemical shifts is shown on top.$^3$
The analysis of the CONCA cross peak intensity data suggests the presence of molecular motions in the extra-membranous fragments of ASR. To obtain a more detailed, site-specific view of the backbone order in ASR, 3D DIPSHIFT correlation spectroscopy was used to measure residue specific one-bond dipolar couplings. Four dipolar interactions per residue were measured to characterize the backbone flexibility: \(^{15}\text{N}-^{1}\text{H},^{13}\text{C}_\alpha-^{1}\text{H}_\alpha,^{13}\text{C}'-^{15}\text{N}\) and \(^{13}\text{C}_\alpha-^{15}\text{N}\). Each coupling was measured in a separate 3D experiment consisting of a series of 2D backbone chemical shift correlation experiments, NCA or NCO, recorded as a function of the dipolar evolution time. The dipolar couplings were recoupled using TMREV sequence to probe \(^{15}\text{N}-^{1}\text{H}\) and \(^{13}\text{C}_\alpha-^{1}\text{H}\) interactions, and ZF-TEDOR to measure \(^{15}\text{N}-^{13}\text{C}_\alpha\) and \(^{15}\text{N}-^{13}\text{C}'\) dipolar interactions. Although both recoupling methods reintroduce multiple interactions simultaneously, the dipolar trajectories (i.e., the dependence of cross peak intensities on the dipolar evolution time) are largely determined by the strongest one-bond dipolar coupling of interest. The experimentally measured coupling strengths are reduced by local motions which occur on the time scale on the order of or faster than the reciprocal of the probed interactions. The dipolar order parameters defined as ratios between the experimental dipolar couplings and the rigid limit, \(<S> = \frac{D_{\text{expt}}}{D_{\text{rigid}}}\) track the amplitude of these motions, which are often visualized as a diffusion cone defined by the semi angle \(\theta\) related to the order parameter as \(\langle S \rangle = \frac{1}{2}\cos \theta (1 + \cos \theta)\). 4

As the spectral resolution of 2D NCA and NCO spectra is not sufficiently high in uniformly \(^{13}\text{C},^{15}\text{N}\)-labeled ASR, the data was recorded on alternately labeled ASR samples, 1,3-ASR and 2-ASR. In Figure 3.2 representative 2D NCO and NCA spectra are shown demonstrating resolution and sensitivity of these experiments. Cumulatively, a total of 92 \(^{15}\text{N}-^{1}\text{H}\) and 68 \(^{13}\text{C}_\alpha-^{1}\text{H}_\alpha\) dipolar couplings corresponding to cross peaks with signal-to-noise ratios (SNR) of at least 15 could be measured in these samples. Additionally, a total of 75 \(^{15}\text{N}-^{13}\text{C}_\alpha\) and 50
$^{15}$N-$^{13}$C couplings could be resolved in TEDOR based correlation spectra. These couplings are evenly distributed along the protein sequence, and can be used for the characterization of the dipolar order parameters in ASR.

**Figure 3.2** Representative two-dimensional spectra of ASR collected at 600 MHz. A) 2D NCO spectrum acquired on 1,3-ASR using the pulse sequence in Figure 2.2 with a ZF TEDOR mixing time of 1.33 ms (16 rotor cycles). The first contour is cut at $10\times\sigma$, with each additional level multiplied by 1.2. Spectra for each TEDOR mixing point were collected with 64 scans, with a recycle delay of 2.0 s, resulting in a total experimental time of 7.7 h for each two-dimensional spectrum. B) 2D NCA spectra acquired on 2-ASR with cross-polarization for $^{15}$N/$^{13}$C mixing. Spectrum was collected using the pulse sequence in Figure 2.1A, without TMREV mixing. Spectra for each TMREV mixing point were collected with 40 scans, with a recycle delay of 2.9 s, resulting in a total experimental time of 7.3 h for each two dimensional spectrum. Blue labels represent cross peak assignments for all resolved residues (BMRB entry 18595).

**Figure 3.3A** shows representative $^{15}$N-$^{13}$C$_\alpha$ ZF-TEDOR dipolar buildup curves for S47 located in the transmembrane portion of helix B, and G145, found approximately one turn below the cytoplasmic end of helix E. Similar to most other residues, an N-C$_\alpha$ bond in S47 is rigid with the corresponding order parameter close to unity. G145 shows slightly slower buildup, but
overall is quite rigid as well, with the best fit order parameter of 0.84. Similarly, the trajectories for $^{15}\text{N}-^{13}\text{C}^\alpha$ peptide bond dipolar couplings for the TM residue Y51 found in helix B, and A152 located in the E-F loop exhibit very similar time scale of the TEDOR buildup (Figure 3.3B).

**Figure 3.3** Representative dipolar trajectories measured in 3D DIPSHIFT experiments. All data were recorded at 600 MHz, using 12 kHz magic angle spinning in TEDOR experiments, and 8 kHz in TMREV measurements. (A,B) Experimental 3D TEDOR buildup curves (open circles) and best fit simulations (solid lines) for (A) $^{15}\text{N}-^{13}\text{C}_\alpha$ and (B) $^{15}\text{N}-^{13}\text{C}'$ couplings. (C,D) Experimental 3D TMREV dephasing curves (open circles) and best fits (solid lines) for (C) $^1\text{H}-^{15}\text{N}$ and (D) $^1\text{H}-^{13}\text{C}$ couplings. TMREV was implemented with 4 MREV elements per rotor cycle. Additional details of simulations and best fits can be found in section 2.2.

Representative TMREV dephasing curves are shown in Figure 3.3C,D for residues S115 and C134 located in helices D and E, and for A64 in the B-C loop. Both S115 and C134 residues exhibit fairly restricted motions on the submicrosecond time scale, with average order parameters of 0.97. On the other hand, N-H and $C_\alpha$-H bonds of A64, which is located in the
region connecting two short β-strands, V61-A63 and Q66-A68, undergo motions of larger amplitudes, with both $<S_{\text{CH}}>$ and $<S_{\text{NH}}>$ average order parameters of 0.89.

Detailed residue-specific analysis of the 3D NH and CH TMREV DIPSHIFT experiments is shown in Figure 3.4. Quite surprisingly, it indicates that all residues in the protein exhibit motions of similar and small amplitudes on a submicrosecond time scale.

**Figure 3.4** One-bond order parameters for $^1\text{H}$-$^15\text{N}$ (A), $^1\text{H}_\alpha$-$^13\text{C}_\alpha$ (B), $^{15}\text{N}$-$^{13}\text{C}_\alpha$ (C) and $^{15}\text{N}$-$^{13}\text{C}'$ (D) couplings as functions of residue number. For each of the measured couplings, the dipolar order parameters were defined as ratios $<S> = D_{\text{expt}}/D_{\text{rigid}}$, where $D_{\text{expt}}$ are experimentally determined dipolar couplings, and $D_{\text{rigid}}$ represents the rigid limit calculated using bond lengths of 1.01 Å for N-H, 1.10 Å for $\text{C}_\alpha$-$\text{H}_\alpha$, 1.46 Å for N-$\text{C}_\alpha$, and 1.33 Å for N-$\text{C}'$.

The $<S>$ values show quite featureless profile for both C-H and N-H bonds (Figure 3.4A-B), with the majority of residues exhibiting the dipolar order parameters in the range between 0.9 and 1.0 for both the $\text{C}_\alpha$-$\text{H}_\alpha$ and N-H bonds, indicating very restricted motions on the submicrosecond time scale for the entire backbone. On average, the extra-membranous regions appear to be slightly more mobile, for example for B-C, F-G and A-B loops.
Similar pattern is observed in the Cα-N effective coupling strengths, which are sensitive to submillisecond motions of the backbone. The majority of Cα-N order parameters fall in the range between 0.90 and 1.05. Order parameters greater than 1 are due to the NCA bond length used to calculate the rigid limit dipolar coupling. The lower <S> values are observed in the A-B loop, and for G145 (<S>=0.84) and T154 (<S>=0.72) in the E-F loop. An increased backbone mobility for these residues correlates well with the CONCA intensities shown in Figure 3.1B, where there is an observed local minimum at the position of G145, and miss correlation corresponding to T154. The C'-N peptide bond order parameters are on average narrowly clustered in the range 0.8 to 0.9, indicating little variation in the peptide bond geometry across the protein, while an overall reduction likely being related to the systematic error in the peptide bond length used for the calculation of the rigid limit.

3.2 Probing slow dynamics by R1ρ measurements

To measure the extent of motions, the 15N transverse relaxation rate in the rotating reference frame was measured site-specifically. The NMR spin-lattice relaxation R2 or the spin-lattice relaxation in the rotating frame, R1ρ, are sensitive reporters on motions occurring on a slow, nanosecond-millisecond time scale.

Site-specific R1ρ measurements were performed at four spinlock power levels (10 kHz, 12 kHz, 14 kHz, 16 kHz). Figure 3.5A shows representative NCA correlation spectrum of UCN ASR. Because of lower sensitivity and limited resolution intensities for a total of 53 residues in the protein were reliably extracted and used to estimate relaxation rates.
Figure 3.5 Representative 2D NCA correlation spectrum of UCN ASR(A). Representative $R_{1\rho}$ trajectories and best fits for A71 located on the extracellular side of helix C ($R_{1\rho}=2.3$ s$^{-1}$), K60 located in the unstructured part of the B-C loop ($R_{1\rho}=5.3$ s$^{-1}$), and Q66 found in the beginning of the second β-strand within the B-C loop ($R_{1\rho}=13.0$ s$^{-1}$). (B) Representative $R_{1\rho}$ trajectories and best fits for T170 ($R_{1\rho}=3.5$ s$^{-1}$) and Y171 ($R_{1\rho}=2.4$ s$^{-1}$) located on the extracellular side of helix F as well as Q195 ($R_{1\rho}=11.0$ s$^{-1}$) located in the F-G loop. (C) The spectrum was collected at 800 MHz, and at a spinning frequency of 50 kHz. 296 transients were collected for every $t_1$ point, with a recycle delay of 600 ms (1.3$T_1$, where bulk $T_1$ was estimated to be 460 ms). Acquisition length in the indirect $^{15}$N dimension was 11.6 ms, and a total experimental time for a single 2D spectrum was 10.8 h. Spectra were processed in two ways. Lorentzian-to-Gaussian-window function with line narrowing of 80 Hz and line broadening of 40 Hz in the direct dimension with 90 Hz line narrowing and 40 Hz of line broadening in the indirect dimension was used to increase signal to noise of isolated cross-peaks. Lorentzian-to-Gaussian-window function with line narrowing of 70 Hz and line broadening of 40 Hz in both the direct and indirect dimension was used to increase resolution of the overlapped peaks (shown as an inset). Red labels indicate resonances that could only be resolved with the second type of processing.

The $R_{1\rho}$ relaxation rates vary dramatically between the transmembrane and exposed regions. While typical $R_{1\rho}$ rates found for TM regions are up to 4 s$^{-1}$, much higher values were measured for the B-C and F-G loops as well as for a few isolated residues at the flanks of α-
helices. Remarkably, an order of magnitude increase in $R_{1\rho}$ was observed for residues in the B-C loop (residues G59-K60, E62-Q66, A68-H69, **Figure 3.6A**), indicating the presence of additional slow time scale motions, which dominate stochastic fluctuations responsible for the transverse relaxation processes. Likewise, highly elevated $R_{1\rho}$ values were found for I185, S188, and G189 in the extracellular F-G loop, indicating additional slow motional regime for this loop as well. Faster relaxation was also observed for E36 ($R_{1\rho}=10.5 \text{ s}^{-1}$) and N148 ($R_{1\rho}=9.5 \text{ s}^{-1}$) located in the cytoplasmic flanks of helices B and E, respectively, again indicating that the A-B and E-F loops may be subjected to slower motions.

**Figure 3.6** Site-specific $^{15}\text{N}$ longitudinal relaxation in the rotating frame measurements. (A) and motional correlation time (B). (A) $R_{1\rho}$ relaxation rate constants determined at 12 kHz spinlock power and (B) motional correlation time estimated using single exponential autocorrelation function. Error bars define 95% confidence level interval. Arrows indicate cases where the lower or upper bound on the correlation time could not be extracted.
Correlation times estimated from **Equation 2.10** are shown in **Figure 3.6B**. Although for the majority of residues only the lower bound on the correlation time could be estimated (due to very high order parameters and relatively large error bars on the $R_{1\rho}$ and the N-H bond order parameter), it was establish that the time scale of slower motions of the B-C and F-G loops are on the order of hundreds of nanoseconds.

**References**


Chapter 4 Conclusions

The conformational flexibility of the 7TM photoreceptor *Anabaena* Sensory Rhodopsin has been studied using SSNMR. We used three-dimensional dipolar chemical shift correlation spectroscopy to determine the effective one-bond dipolar order parameters and characterized amplitudes of submicrosecond time scale motions in the protein. Surprisingly, despite significant variations in cross peak intensities observed in the dipolar correlation experiments, the $^{15}\text{N}-^{1}\text{H}_N$ and $^{13}\text{C}_\alpha-{^{1}\text{H}_\alpha}$ backbone dipolar order parameters indicate that both the TM and exposed regions of the protein undergo only restricted motions on the submicrosecond time scale. Although more variation is detected in the $^{13}\text{C}_\alpha-{^{15}\text{N}}$ and $^{13}\text{C}'-{^{15}\text{N}}$ effective dipolar couplings, which characterize molecular fluctuations of the $\phi$ and $\omega$ torsion angles, these motions appear quite restricted for the majority of residues as well.

In addition, $^{15}\text{N}$ spin-lattice rotating frame relaxation rates were measured under fast MAS to probe slow molecular motions in ASR. Relaxation rates found for the B-C loop on the extracellular side are an order of magnitude greater than the values detected for the transmembrane regions, and are dominated by motions occurring on a time scale of tens to hundreds of nanoseconds. The B-C loop forms short but well defined antiparallel $\beta$-hairpin in lipids. While the local motions of the N-H and C-H bond vectors are likely limited by the intersheet hydrogen bonds, the observed reduction of the order parameters for the residues within this loop are likely due to the collective rigid body motion of this fragment. Although we cannot estimate the amplitude of this motion, it appears that it does not result in effective averaging of dipolar interactions between $^{15}\text{N}$ and $^{13}\text{C}$ spins in cross polarization experiments (Figure 3.1B) as well as between the backbone and side chain $^{13}\text{C}$ spins in rotational resonance distance measurements. It must be noted that the motional propensity of the B-C loop appears to be high.
in crystals where the B-C loop could not be observed due to disorder (or lack of electron
densities). Another extracellular loop connecting helices F and G is also characterized by faster
relaxation rates dominated by slow motions occurring on a time scale of hundreds of
nanoseconds. This loop may act as a hinge point to the outward tilting of helix G under
illumination observed previously using H/D exchange experiments.

Previous solid-state NMR investigations of 7TM microbial rhodopsins show a
complicated picture of dynamics in this type of proteins. In general terms, loop regions directly
interacting with water are more mobile than the intramembrane regions, constrained by well
defined helical secondary structure. The degree of mobility varies greatly from one protein to
another. For example, while most solvent exposed residues in the loops of bacteriorhodopsin
(BR) could be detected in through-space dipolar correlation experiments, and many could be
assigned, the through-bond excitation was required for the detection of the same types of
residues in Sensory Rhodopsin II (NpSRII), suggesting higher degree of loop mobility in the
Sensory Rhodopsin II. Some of the residues in the loops and in the solvent exposed flanks of
helices could not be observed in proteorhodopsin (PR) using the dipolar correlation
spectroscopy, but could be excited more efficiently using scalar driven spectroscopy. Solid-
state NMR measurements on bacteriorhodopsin indicated large amplitude motions in the BC
loop, while EPR studies suggested a time scale of ~200 ns.

Although the sparsity of the data prevents any quantitative conclusions about the time
scale of motions on the cytoplasmic side, we do note the presence of double conformers for a
number of residues detected earlier (Figure 1.1). The chemical shift differences between
different conformers detected in A91, M92 and I95 at 800 MHz proton field range from 0.2- 0.8
ppm for $^{13}$C (40 Hz – 160 Hz), and up to 0.8 ppm for the backbone nitrogens (~60 Hz). No
cross peaks due to chemical exchange could be observed in previously published proton driven spin diffusion experiments\textsuperscript{1,11} indicating that the chemical exchange processes are slower than hundreds of milliseconds.

We demonstrated that SSNMR can be used to site specifically determine the order parameter and time scale of motions in a membrane protein. The results show evidence of slower motions in the inter-helical loop regions of ASR as compared to the transmembrane regions and may suggest the presence of collective motions in the BC loop region where the structure is locally constrained by two antiparallel beta strands. This may aid in the characterization of interactions between the BC loop of ASR and the surrounding extracellular environment about which little is currently known. Additionally the observed mobile antiparallel beta-sheet structures in the BC loop of other microbial rhodopsins suggests that this may be an important characteristic of microbial rhodopsins.

References


