# Chapter **8**

Analysis of build-up curves of cross-peak intensities in <sup>15</sup>N-<sup>15</sup>N PDSD spectra

#### 8.1 Introduction

The solid-state NMR methodology for structural investigation of biomolecules presented in the previous chapters was mainly based on the proton-driven spin diffusion method for the detection of <sup>13</sup>C-<sup>13</sup>C long-range correlations. To convert these correlations into distance restraints, we have used an empirical approach (see Chapter 5). In this Chapter, we want to investigate in more detail the underlying mechanism of spin-diffusion. To simplify the analysis, we investigate the magnetization transfer between <sup>15</sup>N nuclei since the nitrogen spins form a less densely coupled network than the carbon spins. In addition, dipolar truncation effects between nitrogens do only play a very marginal role.

Spin-diffusion processes caused by simultaneous spin flips are of central importance in a multitude of relaxation processes. Spin-diffusion is used to obtain information such as structural heterogeneity, domain size or phase-transitions from a variety of systems like polymers, peptides or polycrystallites<sup>1-6</sup>. The dipolar interactions between proton spins allow simultaneous spin flips (known as 'flip-flops') of pairs of coupled nuclei. Flip-flops between successive pairs of <sup>1</sup>H nuclei in solids provide a mechanism by which magnetization can be transferred through the sample<sup>7</sup>. Spin exchange between rare nuclei such as <sup>13</sup>C or <sup>15</sup>N can occur when these nuclei are coupled to a reservoir of abundant spins, mostly the <sup>1</sup>H. Since the energy transitions in flip-flop processes involved in spin-diffusion are induced by dipolar interactions, it should be possible to relate the distances between low- $\gamma$  nuclei mediated by couplings with the proton reservoir, equations have been proposed to correlate the spin-

exchange rate with the <sup>15</sup>N-<sup>15</sup>N or <sup>13</sup>C-<sup>13</sup>C dipolar coupling constant and hence with the internuclear spin distance<sup>4,8,9</sup>. In one of these equations<sup>8</sup> the spin-diffusion rate ( $W_{ij}$ ) is given in terms of the degree of overlap of the corresponding resonance signals of the spins i and j

$$W_{\rm ij} = (1/2) \pi b^2_{\rm ij} F_{\rm ij} (0),$$

where  $b_{ij} = (1/2) \gamma_S^2 r_{ij}^{-3} (1 - 3 \cos^2 \theta_{ij})$ ,  $r_{ij}$  is the internuclear distance between the i<sup>th</sup> S and the j<sup>th</sup> S' spins,  $\theta_{ij}$  is the angle between the internuclear vector and the direction of the applied magnetic field,  $\gamma_S$  the giromagnetic ratio of S and S' nuclei,

$$F_{ij}(0) = \int_{-\infty}^{+\infty} f_i(\omega - \omega_i) f_j'(\omega - \omega_j') d\omega.$$

The function  $f_i(\omega)$  and  $f'_j(\omega)$  give the resonance line shapes for the signals of the i<sup>th</sup> S and j<sup>th</sup> S' spins.

We have followed a similar strategy to interpret the magnetization transfer in  ${}^{15}N{}^{-15}N$ PDSD experiments recorded on  ${}^{15}N{}^{-16}N{}^{-16}N$ 

In the next Section, the measurement of the <sup>15</sup>N longitudinal relaxation time ( $T_1$ ) of the SH3 amide nitrogens is described. In Section 8.3 the experiment for the measurement of the nitrogen line-width without <sup>1</sup>H decoupling is presented. Finally, in Section 8.4, the relation between build-up curves of cross-peak intensities in PDSD spectra and <sup>15</sup>N-<sup>15</sup>N distances is analyzed.

## 8.2 Measurement of <sup>15</sup>N longitudinal relaxation time

In a peptide backbone, sequential <sup>15</sup>N-<sup>15</sup>N distances range between 2.5 and 3.5 Å, depending on the type of secondary structure in which the nitrogens are located, such as  $\beta$ -sheet,  $\alpha$ -helix or loop. Therefore, the <sup>15</sup>N-<sup>15</sup>N dipolar couplings for these distances are of the order of few tens of Hz. As a result, in PDSD experiments long mixing times of several seconds are required to obtain cross-peaks between two backbone <sup>15</sup>N sites of adjacent residues in a protein. We have measured the longitudinal relaxation time  $T_1$  of the backbone <sup>15</sup>N of the  $\alpha$ spectrin SH3 domain using a 2D <sup>15</sup>N-<sup>15</sup>N experiment recorded at a field of 9.4 T, with the pulse program depicted in Fig. 8.1. The pulse program essentially correlates the <sup>15</sup>N isotropic chemical shifts with high proton decoupling in  $f_2$  with the signal decay due to <sup>15</sup>N longitudinal relaxation in  $f_1$ . The measurement of  $T_1$  is important to estimate the amount of signal that can be lost during the mixing time of the PDSD experiment, due to  $T_1$  relaxation, when the magnetization is stored along the *z*-axis.



**Fig. 8.1** Pulse program for the measurement of <sup>15</sup>N longitudinal relaxation times  $T_1$ . After the CP step, the <sup>15</sup>N magnetization is stored along the *z*-axis, and is then allowed to decay because of  $T_1$  relaxation. After a second 90° pulse, the remaining signal is brought back to the *xy* plane and detected, under TPPM decoupling.

The projection of the 2D along the  $f_2$  axis (Fig. 8.2) shows that the resolution in the direct dimension of the <sup>15</sup>N resonances is quite high, and the <sup>15</sup>N signals can be identified using the chemical shift assignment obtained previously for the  $\alpha$ -spectrin SH3 domain (see Chapter 2). To determine the values of the <sup>15</sup>N longitudinal relaxation times, we have extracted several slices from the 2D spectrum parallel to the  $f_1$  axis and fitted the signals with an exponential function of the type

$$k_1 \exp(-x / k_2)$$
 with  $k_2 = T_1$ 

or with a biexponential function, when the decay of the signal was the result of two different relaxation times.



**Fig. 8.2** Projection along  $f_2$  of the 2D <sup>15</sup>N-<sup>15</sup>N experiment to measure  $T_1$ , recorded at 9.4 T. The assignment of the <sup>15</sup>N signals is reported in the picture and is used to identify the different slices of Fig. 8.3.

In Fig. 8.3 several curves are shown, with the relative fitting. As reported in the figure, the longitudinal relation times of <sup>15</sup>N signals are in the order of 10-40 s. This time is longer than the mixing time used to detect 2D <sup>15</sup>N-<sup>15</sup>N PDSD experiments, hence, signal loss due to longitudinal relaxation during the mixing time is non-significant. However, some loss of signal occurs, which is also reflected by the decrease of intensity of the diagonal signals in PDSD spectra for longer mixing times.



**Fig. 8.3** Slices along the  $f_1$  axis of the 2D <sup>15</sup>N-<sup>15</sup>N experiment recorded at 9.4 T to measure  $T_1$ . Each slice is identified by the assignment corresponding to Fig. 8.2 The <sup>15</sup>N signal-decays are fitted with an exponential function and the  $T_1$  values obtained from the fitting are reported in each plot.

# 8.3 <sup>15</sup>N line-width without <sup>1</sup>H decoupling

To obtain an estimation of the nitrogen line-width without <sup>1</sup>H decoupling, we have measured a 2D <sup>15</sup>N-<sup>15</sup>N experiment with the pulse program depicted in Fig. 8.4. Initially, transverse magnetization is created with a cross polarization step. The magnetization is then allowed to

evolve during the period  $t_1$ , when no proton decoupling is applied. The 90° pulses that follow help to provide a clean spectrum by allowing only the desired longitudinal magnetization to be stored in the *xy* plane and be detected with high-power decoupling during the period  $t_2$ . The pulse program essentially correlates the <sup>15</sup>N isotropic chemical shifts with high power decoupling in  $t_2$  with the non-decoupled shifts in  $t_1$ .



**Fig. 8.4** Pulse sequence of the 2D  ${}^{15}$ N- ${}^{15}$ N experiment, modified to measure the  ${}^{15}$ N line-width without  ${}^{1}$ H decoupling. During the first evolution period ( $t_1$ ), no proton decoupling is applied, while during the acquisition period ( $t_2$ ) TPPM decoupling is used.

The spectra were recorded at three different field strength of 9.4 T, 14.1 T and 17.6 T, and are shown in Fig. 8.5 a, b and c, respectively. In the direct dimension, a conventional 1D <sup>15</sup>N MAS spectrum is obtained, showing the nitrogen isotropic chemical shifts. The resolution in the direct dimension of the <sup>15</sup>N resonances of the <sup>15</sup>N-labelled sample is relatively high, although most lines result from overlapping resonances.



**Fig. 8.5** Contour plots of the 2D  ${}^{15}N{}^{-15}N$  spectra used to measure the non-decoupled  ${}^{15}N$  line-width, measured at 9.4 T (a), 14.1 T (b) and 17.6 T (c). It is interesting to notice the strong effect of the external magnetic field on the line-width: the non-decoupled  ${}^{15}N$  lines are clearly much sharper at higher field strengths.

The  $f_1$ -projection of the 2D experiment recorded at 9.4 T (Fig. 8.5a) is shown in Fig. 8.6. The <sup>15</sup>N signals were assigned using the chemical shift assignment obtained earlier for the  $\alpha$ -spectrin SH3 domain (see Chapter 2). The relative intensities of the different signals is about proportional to the number of spins that contribute to the lines.



**Fig. 8.6** Projection along  $f_2$  of the 2D <sup>15</sup>N-<sup>15</sup>N experiment (Fig. 8.5a) to measure the non-decoupled <sup>15</sup>N linewidth, measured at 9.4 T. The assignment of the <sup>15</sup>N signals is also reported in the picture and is used to identify the different slices of Fig. 8.7.

Several columns extracted from the 2D spectrum of Fig. 8.5a are shown in Fig. 8.7. The different slices are labelled with the relative assignment and the width of the non-decoupled <sup>15</sup>N lines is also reported in the Figure. The non-decoupled <sup>15</sup>N lines can be best fitted with lorentzians with line-widths ranging from 250 to 450 Hz. The exceptions are the proline signals which do not have a NH proton and therefore less broad (see last column in Fig. 8.7). Similar considerations can be made for columns extracted from the 2D spectra of Fig. 8.5b and c. At the higher magnetic fields, the <sup>15</sup>N signals without proton decoupling are clearly sharper. At 14.1 T, the <sup>15</sup>N line-widths are in the 170-230 Hz range, while at 17.6 T they are between 70-150 Hz.



**Fig. 8.7** Slices extracted from the 2D  ${}^{15}N{}^{-15}N$  experiment recorded at 9.4 T (Fig. 8.5a), to measure the non-decoupled  ${}^{15}N$  line-width. In the figure the assignments and the widths of the non-decoupled  ${}^{15}N$  lines are reported.

## 8.4 Build-up curves of cross-peak intensities in <sup>15</sup>N-<sup>15</sup>N PDSD spectra

We have measured a series of 2D  $^{15}$ N- $^{15}$ N PDSD spectra with different mixing times and at different field strengths on  $^{15}$ N-labelled SH3, to study the relation between the build-up curves of cross-peak intensities, the  $^{15}$ N- $^{15}$ N distances and magnetic field strnghts. In Fig. 8.8, cross-peak intensities are plotted as a function of the mixing time. Fig. 8.8a shows build-up curves recorded at 9.4 T, Fig. 8.8b corresponds to measurement at 14.1 T and Fig. 8.8c at 17.6 T. As a guide to the eye, curves corresponding to short  $^{15}$ N- $^{15}$ N distances of 2.6±0.2 Å are depicted in red, while the ones corresponding to long distances of 3.5±0.2 Å are shown in blue. These distances correspond to transfer between  $^{15}$ N in loop regions and in the  $\beta$ -sheet, respectively.



**Fig. 8.8** Build-up curves of cross-peak intensities in  ${}^{15}N{}^{-15}N$  PDSD spectra recorded at different field strengths of 9.4 T (a), 14.1 T (b) and 17.6 T (c).

On the right side of the figure, a detailed legend of the evaluated cross-peaks is included, with in parenthesis the <sup>15</sup>N-<sup>15</sup>N distances measured in the x-ray structure<sup>11</sup>. An evaluation of the

cross-peak intensities showed that there is no straightforward relationship between build-up rates and signal intensities on the one hand, and the distances on the other hand. Only at the lower field of 9.4 T (Fig. 8.8a), the two groups of build-up curves (red and blue) are separated in slow and fast-raising curves. This is not the case at the higher fields of 14.1 T (Fig. 8.8 b) and 17.6 T (Fig. 8.8c). This is expected since the chemical shift difference also affects the cross-peak intensity and should therefore be taken into account. Furthermore, the line-width decreases with the field strength. The magnetization transfer between two low- $\gamma$  nuclei during a PDSD mixing sequence can be explained as an effect of broadening of the resonances in such a way that there is some overlap between the two signals. The broadening is achieved by switching off the proton decoupler during the mixing. Since the amount of overlap depends both on the lineshape and the chemical shift separation, the build-up curves should be scaled with a correction factor that takes into account the chemical shift difference as well. This correction factor is directly related to the overlap-integral of the two interacting resonances. As an outcome of the measurement of the <sup>15</sup>N line-width with no <sup>1</sup>H decoupling (see Section 8.3 and Fig. 8.7), it appears that at each field strength the <sup>15</sup>N signals have similar line-width. intensity and shape. This allowed us to approximate all the signals as lorentzian lines, with an average width that is a function only of the magnetic field strength, assuming that each spin contributed with the same intensity to the spectrum. With these assumptions, our correction factor can be derived analytically, by simply integrating the overlap of two lorentzian lines with line-width  $2\lambda$ , separated by  $\Delta$  (expressed in Hz). A lorentzian function is defined as:





**Fig. 8.9** Schematic representation of two lorentzian lines separated by  $\Delta$  and with a line-width of  $2\lambda$ . The overlap region is coloured in grey.

To calculate the overlap integral between two lines (grey region of Fig. 8.9) we took one line centred at  $\omega_0 = 0$  and the other one at  $\omega_0 = \Delta$ . The overlap integral is 2 times the integral

$$\int_{\Delta/2}^{+\infty} \frac{\lambda}{\lambda^2 + \omega} d\omega = \operatorname{atan} \frac{\omega}{\lambda} \Big|_{\Delta/2}^{+\infty} = \pi/2 - \operatorname{atan} \frac{\Delta}{2\lambda},$$

leading to a correction defined as

 $1 / \{ [\pi - 2 \operatorname{atan} (\Delta / 2\lambda)] / \pi \}.$ 

Hence, the correction factor at 9.4 T, considering an average lorentzian line with width of 350 Hz, can be expressed as

 $1 / \{[\pi - 2 \operatorname{atan} (40.5 * \delta / 350)] / \pi\},\$ 

and is shown in Fig. 8.10 as a function of the chemical shift difference  $\delta$ , expressed in ppm.



Fig. 8.10 Plot of the correction factor at 9.4 T as a function of the chemical shift difference  $\delta$  in ppm.

At 14.1 T, the non-decoupled  $^{15}$ N lines can best be approximated with a lorentzian line with a width of ~200 Hz. The correction factors for this field strength are calculated using the expression

$$1 / \{ [\pi - 2 \operatorname{atan} (60.8 * \delta / 200)] / \pi \}$$

Likewise, at 17.6 T, the non-decoupled <sup>15</sup>N lines can best be approximated with a lorentzian line, but with a width of  $\sim$ 100 Hz. The correction factors are

$$1 / \{ [\pi - 2 \operatorname{atan} (76.0 * \delta / 100)] / \pi \}.$$



**Fig. 8.11** Build-up curves of cross-peak intensities in  ${}^{15}N-{}^{15}N$  PDSD spectra recorded at different field strengths of 9.4 T (a), 14.1 T (b) and 17.6 T (c). The curves are scaled according to the correction factors, as defined in the text.

In Fig. 8.11, the build-up curves are scaled according to the correction factors defined above. For the relatively low field of 9.4 T, the influence of the chemical shift difference is only moderate and the "blue" and "red" curves corresponding to the two classes of <sup>15</sup>N-<sup>15</sup>N distances are already separated, without the need for a correction factor. For the build-up curves recorded at 14.1 T and 17.6 T, the application of the correction factor results in a clear separation of the build-up curves into groups of fast and slowly raising curves, correlating with the <sup>15</sup>N-<sup>15</sup>N distances. In particular, for the measurement at 17.6 T we find a more pronounced relationship between build-up rate of signal intensities and the distances by introducing the contribution of the chemical shift difference.

#### 8.5 Conclusions

We have measured build-up curves of cross-peak intensities in <sup>15</sup>N-<sup>15</sup>N PDSD spectra and scaled the curves, taking into account the chemical shift separation. The slope of the curves can be used to estimate the distance in a simple way. Our approach breaks down in the case that the line-widths are very broad ( $\lambda >>\Delta$ ). However, this is not the case for the spectra recorded on the <sup>15</sup>N-SH3 sample, where the lines are not very broad and, most importantly, have comparable line-width. Furthermore, we do not take into account the effect of MAS in our approach, which can have a strong influence on the signal intensities around the spinning side-band correlations due to rotational-resonance recoupling. However, since only <sup>15</sup>N signals are analyzed that fall in the window between the centre-band and the first side-band the effect of MAS can be ignored.

#### 8.6 Materials and methods

#### 8.6.1 Sample preparation

The SH3 protein was expressed and purified as described in Chapter 2.4.1<sup>12</sup>. For the <sup>15</sup>N-SH3 sample, labelled <sup>15</sup>NH<sub>4</sub>Cl was added to the medium.

#### 8.6.2 Solid-state NMR spectroscopy

The series of 2D <sup>15</sup>N-<sup>15</sup>N PDSD experiments and the 2D <sup>15</sup>N-<sup>15</sup>N experiments for the measurement of the non-decoupled <sup>15</sup>N line-width were performed at 280 K and with a spinning frequency  $\omega_R/2\pi = 8.0$  kHz. For the measurement at 9.4 T we used a wide-bore DMX-400 spectrometer (Bruker, Karlsruhe, Germany). For the measurement at 14.1 T, we used a wide-bore DMX-600 spectrometer (Bruker, Karlsruhe, Germany). Both spectrometer were equipped with 4 mm triple-resonance CP/MAS probes (Bruker, Karlsruhe, Germany). For the measurement at 17.6 T, we used a narrow-bore DMX-750 spectrometer (Bruker, Karlsruhe, Germany), equipped with a 4mm double-resonance CP/MAS probe (Bruker, Karlsruhe, Germany). The 2D <sup>15</sup>N-<sup>15</sup>N experiment for the measurement of the <sup>15</sup>N longitudinal relaxation time was recorded at 9.4 T, at 280 K and with a spinning frequency  $\omega_R/2\pi = 8.0$  kHz.

All the solid-state data were processed with the XWINNMR software, version 2.6 (Bruker, Karlsruhe, Germany) and subsequently analyzed using the program Sparky, version 3.100 (T.D. Goddard & D.G. Kneller, University of California).

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