Protected: Measurement of backbone 15N relaxation times

Introduction

The major factor limiting determination of protein structure by NMR is the sensitivity as modulated by relaxation time. Hence determinationn of this parameter is a critical preliminary measurement to see if a protein together with a set of solution conditions (eg. temperature) are suitable for structural determination.

Relaxation is a summation of processes by which magnetization decays over time, thus limiting the useful time of observation after exciting with an RF pulse. Relaxation mechanisims are grouped into two classes named T1 and T2. T1 relaxation corresponds to restoration of equilibrium between the numbers of nuclei in the high and low energy spin states. T2 represents loss of phase coherence. The pulse sequences extract structural information by transferring magnetization in a sequence from one nucleus to another and allowing the magnetization to evolve over time at each position. Because each nucleus has a characteristic relaxation time that governs loss of signal while at that nucleus, the relaxation times have to be long enough to allow the pulse sequences to complete and generate a useful signal. The physical mechanisms which determine the ^{15}N relaxation time are understood and can be related to the overall rotational correlation time of the protein (tumbling rate), as well as a component due to internal flexibility of individual segments of the polypetide chain.
Since the T2 relaxation time of ^{15}N is easily measured, its value is taken as a good indicator of the prospects for successful structural determination, as well as an indicator of flexibility in local parts of the protein structure.

Mathematically, T2 is defined by the equation $Mag_{xy}/Mag_{xy\_max} = e^{-t/T2}$, and is measured by fitting the observed loss of magnetization in the xy plane over time to an exponential . ^{15}N T2 of less than 60 msec may be too short to support structural determination. Note that the more elaborate 3D and 4D experiments required in structural determination have longer pulse sequences and are hence more vulnerable to loss of signal by relaxation than are the preliminary HSQC experiments. Hence sensitivity of HSQC per se is not an adequate indicator of feasibility for structural determination, and ^{15}N T2 measurements are recommended at an early stage in the sequence of experiments..

The major factor influencing the T2 is the tumbling rate of the protein. Rapid tumbling gives long T2's, whereas slow tumbling gives short T2's. The mechanism is that tumbling generates oscillating electromagnetic fields; slow tumbling happens to generate fields in the right frequency range to more effectively interact with the fields of the magnetized nuclei and exchange energy. The tumbling is in turn governed by the size of the protein. This gives the characteristic limitation of NMR to work on small proteins or protein fragments. In addition to the size of the protein, the shape also governs the mode of tumbling. However, one is not guaranteed to have useful T2 times just by having a protein below a certain molecular weight. In addition to the size of the protein, the shape also governs themode of tumbling. Also, self-association (weak forms of aggregation) may cause the protein to behave on average as if it is larger than a monomer. Often one can infer information about protein self-association from the T2 measurements.

If the target protein gives marginal T2 times, then possible remedies are 1) raise the temperature (improves tumbling through reduced solvent viscosity), 2) lower pH (increases sensitivity through decreased hydrogen exchange), 3) adjust solution conditions to prevent aggregation, 4) dueterate the protein (attenuating some relaxation pathways; note that the amide hydrogens themselves will be exchanged with ^{1}H prior to the NMR experiment, hence the experiment is still a $^{1}H/^{15}N$ HSQC measurement), 5) use a NMR machine with a higher field strength (more sensitivity), or 6) engineer a smaller protein fragment.

The T2N15 measurement

The experiment is a series of HSQC measurements modified so that the magnetization will remain on the the nitrogen a different amount of time in each measurement. The delay time during which the ^{15}N relaxes is specified by the parameter l4 (L4), which you will set differently for each determination in the series. The total time of this series of measurements will be about 48 hours (for NS=32 and 11 total determinations). You will

process the spectra like HSQC spectra and then extract the T2 by fitting the decline in signal strength over time to a decreasing exponential.

The instructions given below are phrased as modifications to the basic HSQC acquisition and processing instructions.

Sample loading, locking, shimming, tuning

Same as for HSQC.

Pulse Program and dataset

Acquire T2N15.ref pulse program and dataset. Set up experiment 1. Since the signal is 10-50% lower in this experiment than an HSQC, the receiver gain (parameter RG) is set higher (512 or 1024 vs. 256 for HSQC), and you may want to increase NS from 16 to 32 if your HSQC has marginal signal intensity. Note RG and NS determine the noise level you can expect during processing. For experiment 1, set up l4 (lower case L4) to be 1 (L4 is in units of 8 msec).

Pulse calibration

Review the pulse program. There are two rectangular pulses to calibrate and determine phases analogous to p1 and p2 of HSQC. Note that the phase difference for p2 must be set on parameter phcor14.

Other parameters

Check that l3 (L3) is 170 and 1 TD = 2*L3. Note for processing that SW in the ^{15}N dimension is 1/(2*in10) and check with *eda* that in10 is 330 usec. Also check that in0 is set to 16 usec, and in9 is set to 314 usec. Note that spectral width in the ^{15}N dimension is a little lower as set above than it is for the standard hsqc experiment. One could therefore detect folded peaks by comparing these two spectra. If you wish to change the ^{15}N spectral width in this experiment, in10 should be adjusted accordingly, and in9 should be adjusted to remain = in10-in0 (in0 stays the same).

Transiently start the first acquisition and look at the first serial file (by *rser 1*).

Water suppression is intrinsically less effective in this sequence, so expect a big signal in the transformed spectrum at the water position. Be particularly alert that the fid is not offscale prior to transformation. This would mean that the remaining water signal is so

intense that there is truncation of the signal. This condition will require improvement of your calibrated pulse times and phases.

Use edc to set up a series of experiments differing only by L4

Review the comments in the pulse program about the T2. Cover the range up to 120 msec (L4=15). If your protein is small, you may want to include some higher values. The experiments will be performed in the order of their experiment numbers. Randomize the order of L4 values within the series so that some other fluctuation over real time will not masquerade as part of the T2 decay. Repeat L4=1 at the end to see if there has been any fluctuation in the sample over the time of the experiment. Record in the title file for experiment 1 the settings for L4 for each experiment number. eg.

Multizg

Multizg is a command that executes a sequence of experiments. Set the dataset to experiment no. 1. Type *multizg*. Answer the question as to how many experiments you want to start. Multizg will indicate the time for the entire series of experiments.

Data processing

Make 2D spectra:

- Copy the entire dataset including all of its experiments to your NIS data directory.
- Process exper no. 1 as for HSQC. SW in the ^{15}N dimension is 1/(2*in10). The water region is likely to retain a water signal, so use *EXT -x1 -xn* to remove this region.
- The phases for the 2nd dimension are both 0.00.
- Copy the conversion script and processing script (fid.com and nmrproc.com) to the subdirectory containing each of the other experiments and execute them so that all experiments are processed exactly the same. eg. *cp fid.com ../2/.*

Select a set of peaks to analyze:

- Go to experno. 1, run *nmrDraw* and view the 2D spectrum.

- Adjust the <first> contour level entry in the control panel to eliminate spurious peaks, but to reveal the smallest peaks of interest.
- <peak><peak detection>
- Fill in peak widths in pixels (typically about 5 pixels) that causes peaks to be picked in the subsequent <detect> operation without undue subdivision.
- <detect>. Leave “index” in the labels box.
- In the Peak Detection window, click <edit>. Using the right mouse button, delete all peaks except for well separated meaningful peaks. On the <peak><peak detection> menu, click <save> to save a table of these peak values named test.tab.

Compare the first and last spectra (which should be the same with 8 msec).

- Visually inspect the first and last 2D plots for appearance/disappearence of specific peaks which may indicate proteolysis or unfolding of the sample over the time of the measurement.
- Use vi to create a file named 12List with the following contents where <expno.> is the number of your last experiment and 2D filename is the final filename output by your nmrproc.com script.
  - *./<2D filename>*
  - *../<expno.>/<2D filename>*
- Be careful not to leave blank lines at the end of the file.
- Run the program seriesTab as follows:
  - *seriesTab -in test.tab -out 12Out.out -list 12List -dx 6 -dy 6*
  - This will tabulate the same peaks from both spectra side by side. –*dx* and -*dy* specify a box of 6 x 6 points that will be centered on each peak to tabulate the volume of the peak.
  - It is convenient to type commands such as the above into a script file, eg. named 12.com. You must issue the command *chmod u+x 12.com* to make the .com file executable. Then you can perform the function just by typing *12.com*. This both saves on typing if you have to repeat the command several times, and also leaves a record of exactly what you did in your directory.
- Examine the table 12Out.out with vi. There should be a series of entries each starting with the peak number, the x and y coordinates in points, many other parameters as indicated by the labels at the top, and ending in 1.0000 followed by a final number. The last number is the relative intensity of the same peak in the last acquisition compared to the first (both at 8 msec if you followed the instructions above). The relative intensity should be close to 1. If there is a systematic decline in

intensity, then you are losing signal over the course of the experiment for some reason, such as protein aggregation or proteolysis.

Tabulate the T2 relaxation time

- Create a list file with vi, eg. seriesList, as above only listing each experiment one per line in the order of increasing L4 delay time.
- Execute seriesTab with this list file. eg: *seriesTab -in test.tab -out seriesOut.out -list seriesList -dx 6 -dy 6*.
- Look at seriesOut.out with vi and notice that the index for each peak is in column 1 and the series of declining intensitites starts in column 26 and goes to the end (35 in my case).
- Use utility *getCols* to extract a simplified table with just these columns, eg. *getCols seriesOut.out cut.out -hdr 0 25 26 27 28 29 30 31 32 33 34*.
  - Note that *getCols* counts its columns from 0 instead of 1.
  - View cut.out with vi to be sure you now have a table with the peak number in column 1, followed by a 1.0000 and then the appropriate number of entries.
- Use vi to strip off the remaining labels at the top of cut.out, and add a row at the top putting the time in msec over each column (separated by spaces). Remember that L4 is in units of 8 msec. This is most conveniently formatted for printing of the table by placing the times directly over each column of measurements with a blank entry over the peak index column.
- Edit the table to remove any rows that are unreasonable, eg.drop from 1.00 to very low numbers at the first point and have negative numbers in them. These are noise peaks that you neglected to remove in processing your picked peaks.
- Count the number of remaining rows (peaks).
- Use the utility *quick* to fit exponential curves to this data as follows:
  - *quick cut.out cut.fit 10 48 2*
  - The first filename is the input file, the 2nd is a new output file.
  - The first numeric parameter is the number of time points, the 2nd the number of rows to process, and the 3rd specifies a 2 parameter fit or a 3 parameter fit.
  - Look at the output file. Each row has 5 numbers. They are
    - 1 the peak index
    - 2 the intercept (should be close to 1.000)
    - 3 the error in the intercept
    - 4 the T2 relaxation time in msec.
    - 5 the error in the T2 relaxation time.

- It is customary to make a histogram showing the distribution of T2 times for your protein.
- After peaks are assigned to specific residues, it is customary to plot T2 by sequence coordinate. This will reveal regions of local high mobility (unusually long T2's).
- If you wish to reassociate specific T2 times with specific peaks on your 2D plot, you can reload test.tab from the <peak detection> window and redraw the index labels on the plot.