Investigation of ubiquitin dynamics using NMR-based relaxation dispersion measurements

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Lab Rotation - III Report

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Summary

The state-of-the-art solution NMR techniques provide the unique ability to traverse the conformational landscape of proteins and provide atomistic details of the various interconverting structures, the rates of interconversion and the free-energy difference between transient states that are sparsely populated at a given temperature. A single experiment (CPMG, $R_{1\rho}$) can provide this wealth of information in a broad range of time scale spanning μs-ms without perturbing the structure of the proteins. Hence, there have been increasing efforts in expanding this scale to be able to visualise the entire spectrum of dynamics exhibited by proteins. These efforts have been made possible due to manifold advances in various aspects, such as methods of protein labelling and expression, instrumentation developments, and devising new pulse programs as discussed in [1].

Ubiquitin has been a protein of interest to study the dynamics at various time scales. Despite being a relatively smaller protein, it shows a range of dynamics and has numerous binding partners. Hence, it is an interesting protein to discover underlying mechanisms of binding and conformational dynamics. We have also used ubiquitin in this project to test the application of E-CPMG, a method developed previously in the lab that now utilizes the CPMG experiment in its full capacity probing a wide range (4μs-10ms) of timescale. Based on some hints from super-cooled CPMG experiments about putative dynamics in the ns-μs range, we wanted to characterize this motion. We planned to combine the high power CPMG experiment along with addition of a viscogen (glycerol) to be able to slow down motions from the ns-μs into the detectable window of our CPMG experiment along with being able to perform solution NMR experiments at reduced temperatures preventing freezing or cold denaturation of certain proteins.

As a first check, we observed that glycerol addition slows down motions in the ps-ns range from a 5-fold increase in the average global τ_c of ubiquitin. The CPMG results from the glycerol containing samples wasn't clean, but based on initial observations we did not observe a uniform slowing down of the peptide flip and the pincer mode motion (identified in the reference sample) based on the idea that glycerol might increase solvent friction and reduce exchange kinetics.

1 Introduction

Our understanding of protein structure-function relationship is largely limited by the static time-averaged three-dimensional picture of proteins in our minds. A complete knowledge of the function of a protein would require understanding of the conformational dynamics between all its thermally accessible states. This is particularly important for characterizing intrinsically disordered proteins (IDPs) that do not have a unique, well-defined ground state, per se. NMR techniques have the unique capacity to capture the dynamics of proteins at various time scales at atomic resolution along with quantifying the rates of interconversion (kinetics) and the population of different states (thermodynamics). In particular, heteronuclear spin relaxation methods are regularly used to detect protein dynamics in the picosecond-nanosecond ($ps - ns$) and microsecond-millisecond ($\mu s - ms$) time scales.

A qualitative idea of the range of dynamics exhibited by proteins is often described by a hierarchical structure comprising slow-interconverting macrostates, which are themselves defined by faster-interconverting microstates as summarized in Fig. 1. Intramolecular motions in the ps-ns time scale can be probed by laboratory frame NMR relaxation techniques. Relaxation in this regime occurs due to the overall rotational diffusion of the molecule and internal dynamics. But, only dynamics that occur faster than the rotational correlation time τ_c (sub-τ_c motion) can be measured in these experiments which account for the conformational entropy in the protein. Biologically more relevant phenomenon such as molecular recognition, enzyme catalysis and protein folding occur in the μs-ms range and therefore it is important to study the sparsely populated states that interconvert in this window. Such states are often referred to as "invisible" excited states as we are just beginning to characterize them using developing NMR methods.

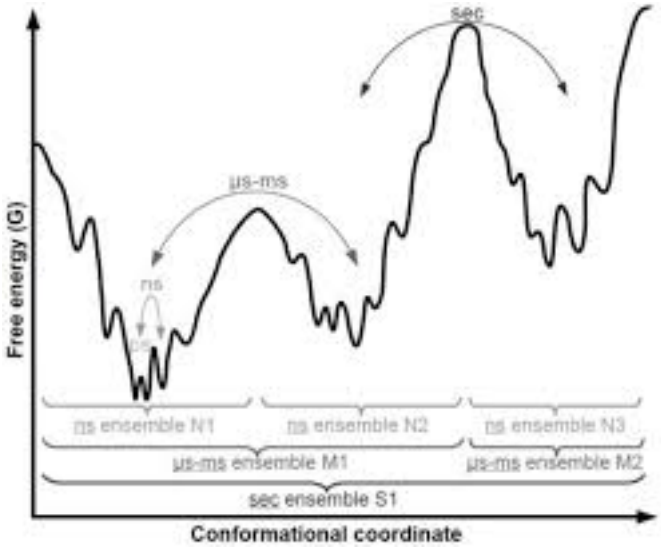

Figure 1: The figure shows the conformational landscape of a particular protein comprising differentially populated states that interconvert in ambient temperature at different time scales. Based on the range of motion probed, one can detect the time-averaged and ensemble-averaged structures - *image taken from* [5]

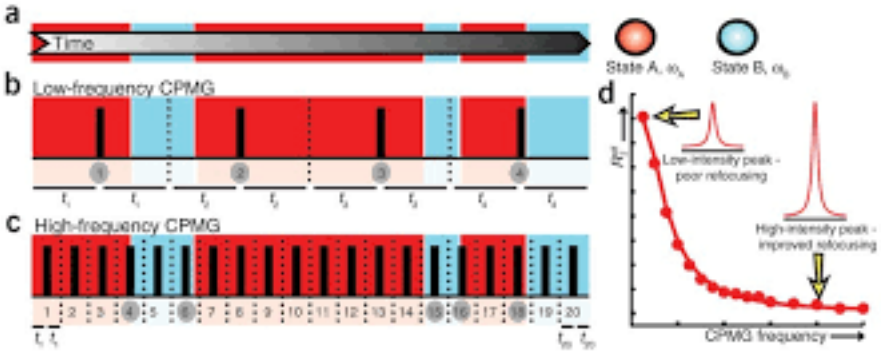

Figure 2: Schematic of a CPMG experiment. An atom switches from state A to B stochastically. Pulses are applied to modulate the relaxation rate and calculate the parameters of the two-state exchange reaction - *image taken from* [2]

1.1 Relaxation dispersion measurements

Relaxation dispersion or equivalently, line broadening of peaks occurs due to chemical (or conformational) exchange processes that take place in the μs-ms range. Basically these processes alter the magnetic environment of the atoms by changing their chemical context in a time-dependent manner within the above time range. This leads to time-dependent resonant frequency changes which cause dephasing of the transverse coherences. Hence, the effective transverse relaxation rate ($R_{2,eff}$) has a contribution from the chemical (or conformational) exchange (R_{ex}) in addition to the intrinsic relaxation ($R_{2,int}$) originating from sub-τ_c motions alone. R_{ex} can be readily measured using relaxation dispersion experiments such as Carr–Purcell–Meiboom–Gill (CPMG) or $R_{1\rho}$ relaxation. Although both experiments are equivalent in some sense, the focus here will be on CPMG experiments that were used in our analysis. Fig. 2 shows a schematic of the CPMG experiment in which a train of refocusing pulses are applied to quench the relaxation component from chemical exchange.

Ubiquitin, which is the focus of this study, is a versatile protein that is recognized by its many binding partners. The solved crystal structures show significant heterogeneity and this could be explained based on its putative dynamics in the supra-τ_c range as discussed in [6]. Hence, there has been interest in probing this window using different NMR techniques. CPMG experiments could only examine motions in the range of 50μs-10ms previously due to the technical limitation of the power that the probehead could sustain. Increasing efforts have been made in the field to expand this window to capture faster motions. A cryogenically-cooled probehead [3] has made it possible to now examine dynamics up to a few μs. This new development that uses higher power is now termed extreme CPMG (E-CPMG) and it enables the measurement of kinetics of both slow and fast dynamics in one simple experiment as demonstrated in [10]. In Ubiquitin, this method helped to determine two different motions called the peptide flip motion and the pincer mode motion that occur in different parts of the protein as reported in [12]

In this project, we want to investigate the effect of a small viscogen, glycerol, on the μs dynamics of ubiquitin. Typically, one uses extremely reduced temperature to slow down

the kinetics in ns-μs range to bring it in the detection window of CPMG experiments. To get rid of any possible artefacts that we observe due to extreme cooling, we want to be able to slow down these motions using a viscogen instead, that putatively doesn't perturb the structure of the protein. This would also allow performing experiments with lower temperatures without freezing the solution and keeping the protein in its intact three-dimensional structure. At the same time, an increase in the rotational tumbling time τ_c due to increased viscosity, could also widen the sub-τ_c spectrum and close in on the "invisible" ns-μs window.

1.2 Two-state exchange models

To fit the two-dimensional heteronuclear (^1H-^{15}N) CPMG relaxation dispersion data describing a two-state exchange, the following models are regularly used. They were implemented here using the scripts developed in the lab based on the web interface of ShereKhan [8]. (A) Bloch-McConnell model [9] is applicable to any exchange regime while (B) Luz-Meiboom model [7] is applicable for the fast regime ($k_{ex} > \delta\nu$) where,

$$R_{2,eff}^{calc}(\nu_{CPMG}) = \frac{1}{T_{CP}} \ln \frac{I(\nu_{CPMG})}{I(0)}$$

$$I(t) = \left(\exp(Rt)\exp(R^* t)\exp(R^* t)\exp(Rt)\right)^n I(0)$$

$$R = \begin{pmatrix} -R_2 - k_{AB} & k_{BA} \\ k_{AB} & -R_2 - k_{BA} + 2\pi i \Delta\delta B_0 \end{pmatrix}$$

$$n = T_{cp}\nu_{CPMG} \qquad t = \frac{1}{4\nu_{CPMG}}$$

(A) Bloch-McConnell model

$$R_{2,eff}^{calc}(\nu_{CPMG}) = R_2 + \frac{\Phi}{k_{ex}}\left(1 - \frac{4\nu_{CPMG}}{k_{ex}} \tanh\left(\frac{k_{ex}}{4\nu_{CPMG}}\right)\right)$$

$$\Phi = 4\pi^2 B_0^2 \varphi$$

(B) Luz-Meiboom model

Figure 3: Models used for two-state fitting adapted from ShereKhan [8]

T_{CP}: given constant relaxation delay
ν_{CPMG} : given CPMG frequency
B_o : given field strength for the nuclei of the interest in MHz
R_2 : fitted intrinsic transverse relaxation rate
k_{AB}, k_{BA} : fitted kinetic rate constants (slow exchange)
k_{ex} : fitted kinetic rate constant (fast exchange)
$\Delta\delta$: fitted chemical shift difference in ppm (slow exchange)
ϕ : $P_A P_B \Delta\delta^2$ (fast exchange)

2 Methods

The sample of ubiquitin that was used in the NMR experiments was provided by Dr. Stefan Becker. The perdeuterated, 15N-labeled WT ubiquitin was expressed in *Escherichia coli* adapted to 100% D2O minimal medium with D7-glucose as a carbon source and 15N-NH4Cl as a nitrogen source. Using this, the following set of mixtures were made and used for the NMR experiments - (i) reference sample of ubiquitin(1.2mM) in phosphate buffer (20mM, pH 6.5) (ii) sample of ubiquitin with 40% v/v d8-glycerol in water (iii) samples of ubiquitin with 20%, 25%, 31.6%, 35.5% d8-glycerol in water used for titration and following peak shifts. The set of NMR experiments that were analyzed in this project were performed by my supervisors, which include -
(a) ^{1}H-^{15}N Transverse relaxation optimized spectroscopy (TROSY) experiments of samples in (i),(ii),(iii) at 277K, 900 MHz
(b) TROSY-based ^{15}N R_1 and R_2 relaxation experiments for samples (i) and (iii) at 277K, 900 MHz
(c) $^{1}H^{N}$ Carr–Purcell–Meiboom–Gill (CPMG) experiment with samples (i) and (iii) at 277K, 950 MHz. The CPMG frequency (ν_{CPMG}) was varied from 1200 to 34,000 Hz and from 400 to 32,800 Hz during a constant time period (T_{CP}) of 20 ms.
Effective relaxation rates ($R_{2,eff}$) were fit assuming fast exchange using the Luz-Meiboom model. NMR data processing was done in Bruker Topspin and peak quantification on CcpNmr Analysis[13] and Cara[4]. Python scripts [8] were used for fitting and plotting of the data.

3 Results

To begin with, I obtained the two-dimensional TROSY spectra of the ubiquitin reference sample (in buffer) and assigned the peaks on CcpNmr Analysis using the already available peak assignement as shown in Fig. 4. 67 peaks were assigned, without any ambiguity, of its 76 residues. 5 residues - 1Met, 19Pro, 36Ile, 37Pro, 38Pro are not seen in the spectra. 2 peaks of 24Glu and 53Gly are missing. 2 pairs of residues are merged, 27Lys with 18Glu and 26Val with 16Glu.

To analyze the effect of addition of glycerol on the overall structure and motion of ubiquitin, TROSY spectra with increasing concentrations of glycerol were recorded. As is apparent in Fig. 5 the effect of glycerol is not random and increases in a systematic manner in the direction of the peak shift. To quantify the chemical shift differences observed upon addition of glycerol, I calculated a parameter termed as chemical shift perturbation index which is defined as follows:

$$CSP = \sqrt{(\Delta^{1}H)^2 + 0.1 * (\Delta^{15}N)^2}$$

CSP was calculated based on the changes in peak positions with respect to the reference sample as is summarized in Fig. 6. The extent of the effect of glycerol on different residues is different, but this could possibly arise due to inherent dynamic nature of some of the residues. To visualise the CSP values with respect to ubiquitin structure, I overlayed the

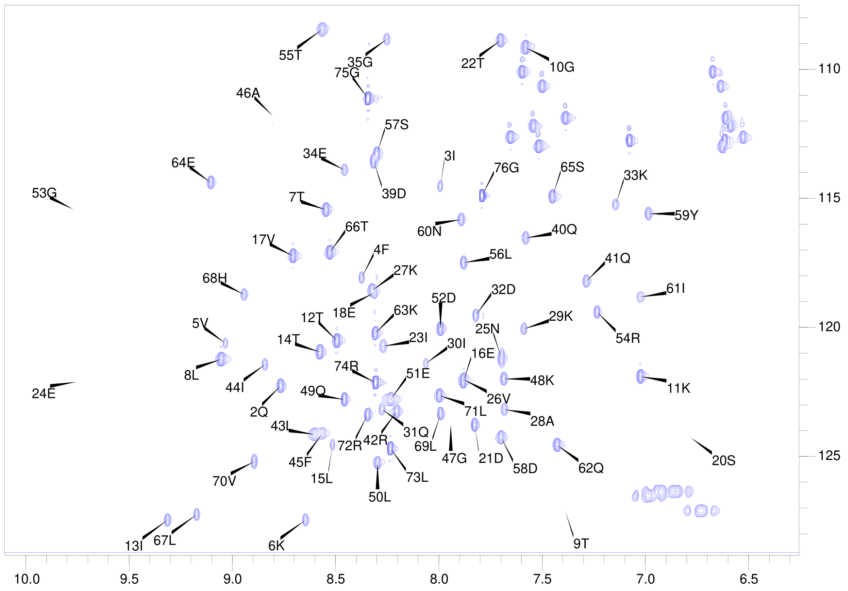

Figure 4: Ubiquitin peak assignment in a ^{1}H-^{15}N HSQC spectrum. Positive peaks are in blue while negative peaks are not displayed but labelled.

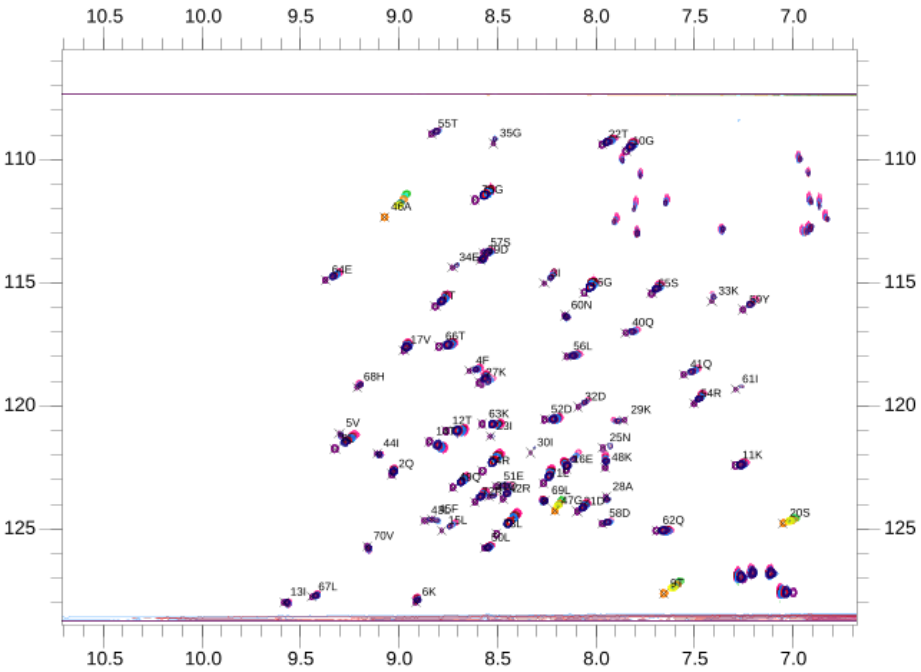

Figure 5: Ubiquitin peak assignment in ^{1}H-^{15}N TROSY spectra with increasing glycerol concentrations.

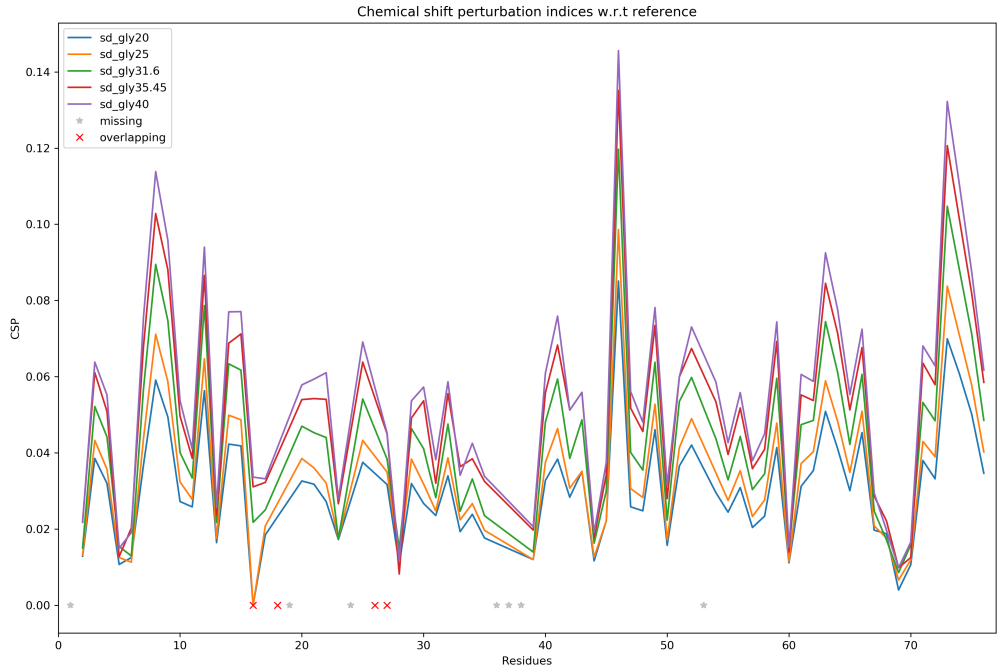

Figure 6: Chemical shift perturbation indices calculated for all the assigned residues of ubiquitin which do not overlap in the ^{1}H-^{15}N TROSY spectra recorded at increasing glycerol concentrations.

values on its crystal structure (PDB: 1ubq) as shown in Fig. 7. The highest values of CSP are seen for the residues 8Leu, 46Ala, 73Leu, 74Arg which are in the loop regions in ubiquitin. It could also possibly mean that the local environment of the backbone of these residues is changing in the presence of ubiquitin and hence this must be carefully looked at in further analyses. These experiments signified that there are no major structural rearrangements or protein unfolding that occurs upon glycerol addition. This was also observed in [11] where the authors used to study the folding kinetics of four-helix bundle FF domain in the presence of glycerol. We hence proceeded to characterize the effect of glycerol on the overall tumbling motion of ubiquitin.

I calculated the τ_c of ubiquitin in the reference sample and in d8-40% glycerol based on the R_1 and R_2 relaxation data assuming that the protein is rigid and spherical. If we assume the protein of interest to be globular, then the rotational correlation time can be calculated as

$$\tau_c = \frac{1}{4\pi\nu_N}\sqrt{6\frac{T_1}{T_2} - 7}$$

where, $T_1 = 1/R_1$ and $T_2 = 1/R_2$. Based on Stoke's law, τ_c is also related to η, the viscosity of the solvent, as

$$\tau_c = \frac{4\pi\eta r^3}{3k_B T}$$

Hence, we would expect the τ_c to increase in glycerol, albeit not linearly, as our protein

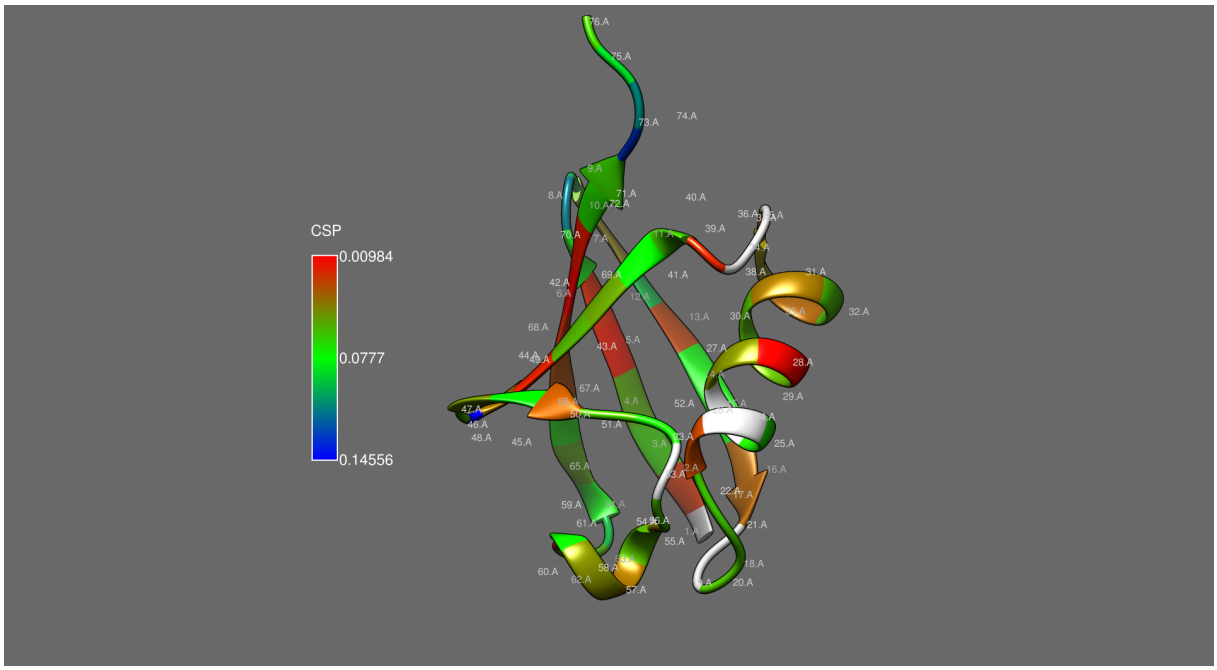

Figure 7: Chemical shift perturbation indices of residues of ubiquitin calculated from the TROSY spectrum obtained at 40% glycerol concentration. CSP values are rendered as attribute for 1ubq.pdb with a linear scale. Residues in white are missing residues with no data.

is neither perfectly rigid nor spherical. The viscosity of glycerol and water differs in 4 orders of magnitude, but this was obviously not so drastic in the difference of observed τ_c. Fig. 8 and 9 summarize the data from the R_1 and R_2 relaxation experiments for each residue and the τ_c calculated using the above formula. To calculate average τ_c only residues comprising secondary structure of ubiquitin were considered to adhere to the rigidity assumption as mentioned above. Based on this, I observed $\overline{\tau_c}$ for the reference sample to be $\sim 8.11 \pm 0.08$ ns , and for the sample containing 40% glycerol to be $\sim 43.7 \pm 5.5$ ns. This indicates the effect of increased viscosity in slowing down the rotational tumbling and a corresponding 5-fold increase in τ_c.

Based on this premise, we wanted to then perform E-CPMG experiments on the glycerol containing ubiquitin sample. A priori, based on the understanding that glycerol could exert an increased solvent friction, we could expect that the motions we already see (peptide flip motion and pincer mode motion in ubiquitin) might show slower rates. Also, based on super-cooled experiments using E-CPMG, we saw hints of some dynamics lying in the faster(ns-μs) regime, which we hoped we could also observe in the glycerol-containing samples. So, we performed the E-CPMG experiment using the reference sample upto $\nu_{CPMG,max}$ of 34 kHz, which would allow us to detect fast motions with a lifetime of $\sim 5\mu$s. The results showed that the relaxation dispersion curves for all the residues lied in the fast regime and hence could be fitted well using the Luz-Meiboom model. A set of residues (Q2, I3, K6, T9, G10, D21, N25, E34, D39, I44, N60, K63, S65) clearly displayed fast-scale motion with a grouped $k_{ex} = 122171.27 \pm 6074.12 s^{-1}$ or equivalently $\tau_{ex} = 8.18 \pm 0.41 \mu s$. Some of the individual fits are showed in Fig. 10. Another set of residues (E51 and I61) as shown in Fig. 11 showed slowest-scale motion with a grouped

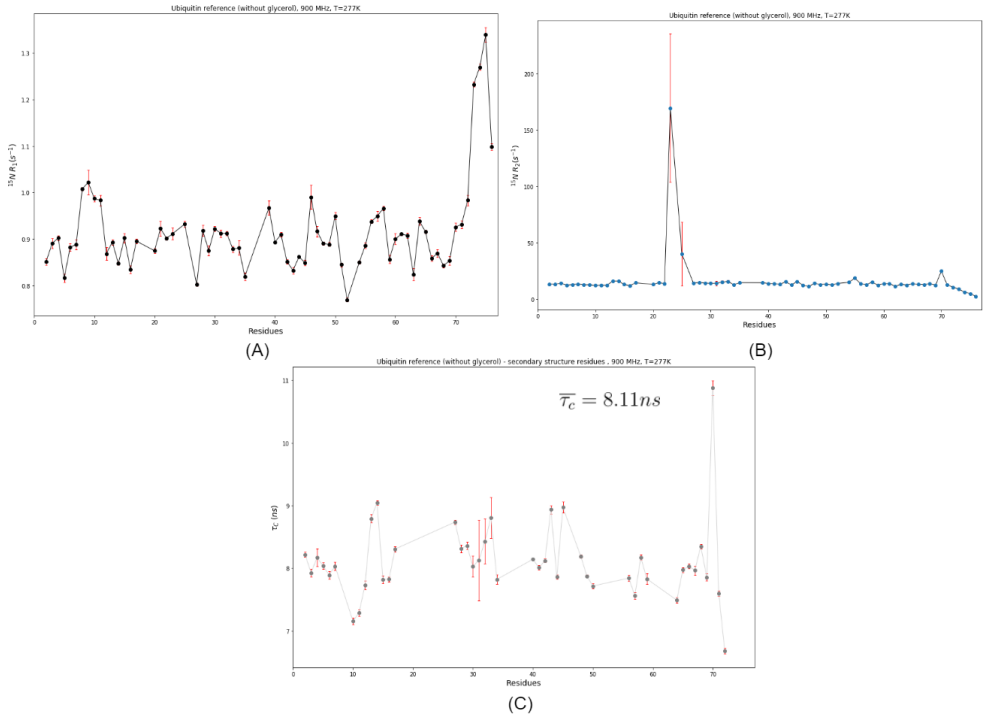

Figure 8: Calculation of τ_c for the ubiquitin reference sample in water at 900 MHz and 277K (A)$^1 5NR_1$ and (B)$^1 5NR_2$ relaxation rates plotted against all residues. (C) τ_c calculated for the residues in defined secondary structure and their average reported

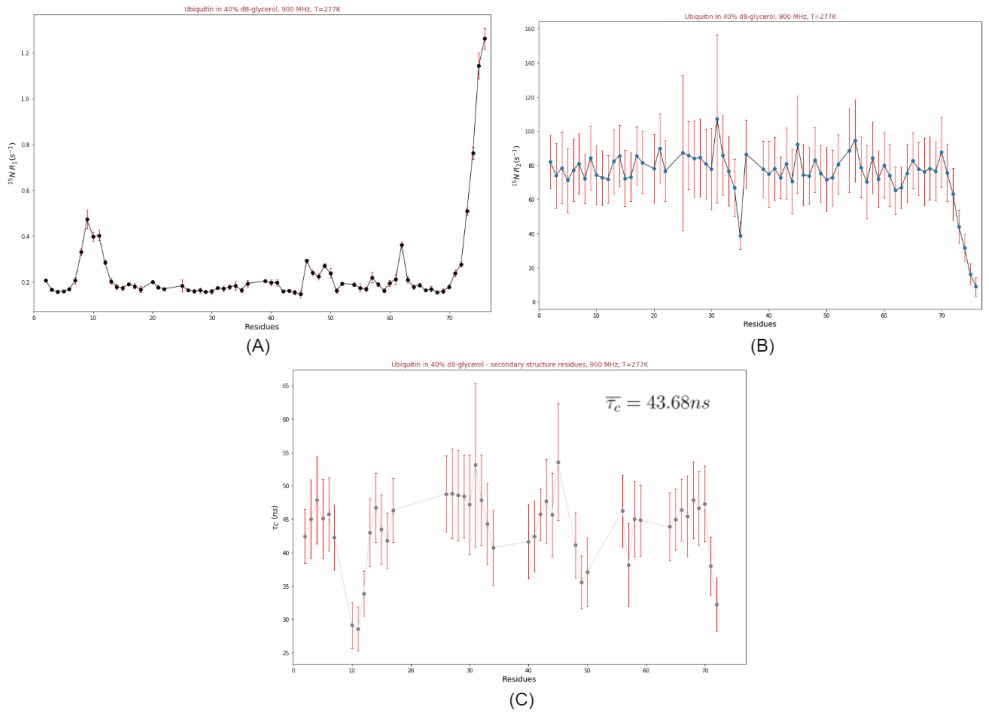

Figure 9: Calculation of τ_c for the ubiquitin sample in 40%d8-glycerol at 900 MHz and 277K (A)$^1 5NR_1$ and (B)$^1 5NR_2$ relaxation rates plotted against all residues. (C) τ_c calculated for the residues in defined secondary structure and their average reported

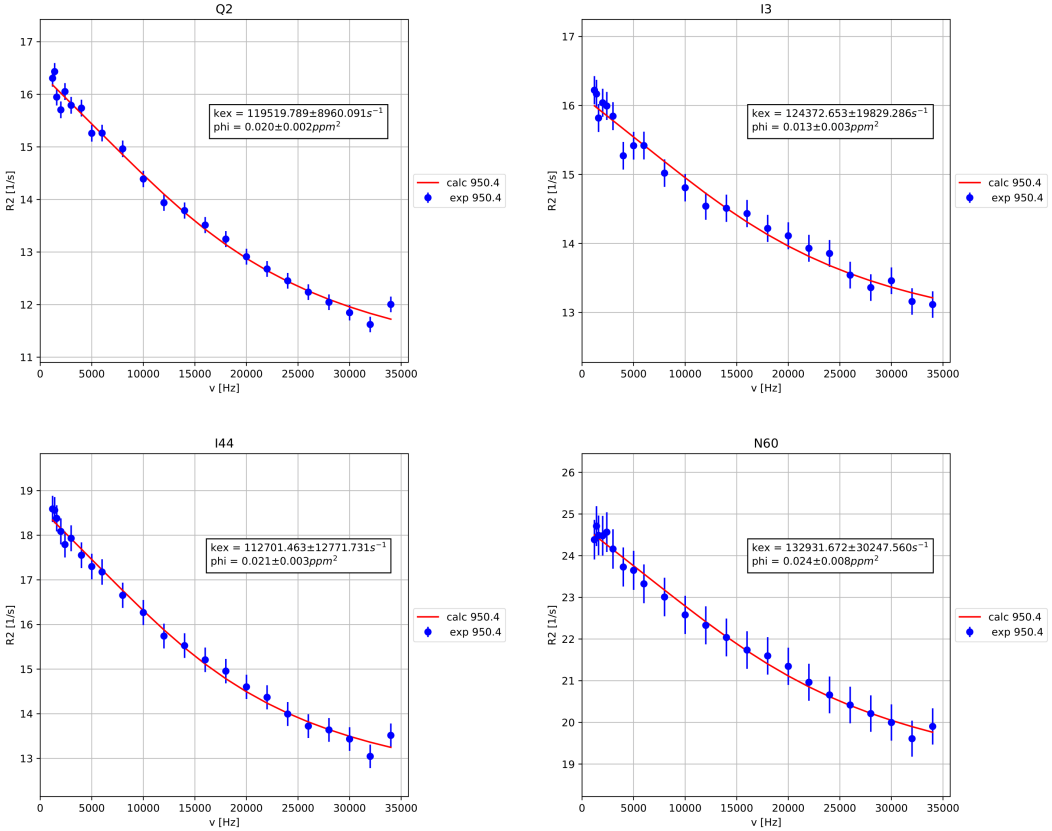

Figure 10: Two-state exchange fitting according to LM model for residues (Q2, I3, I44, N60) in the fastest exchange regime. Based on previous results from the lab, these residues most likely exhibit Pincer mode motion

$k_{ex} = 24246.18 \pm 805.43 s^{-1}$ or equivalently $\tau_{ex} = 41.24 \pm 1.37 \mu s$. But most of the residues couldn't be fit accurately with a simple two-state LM model and indicated that a single k_{ex} does not suffice to explain the exchange kinetics. This implied that at least two distinct modes of motion co-exist and affect the dynamics of most of the residues. The shape of the curves also hinted that the exchange rates of these motions differ significantly and can be decomposed into fast and slow ranges. This analysis couldn't be completed in the duration of the project but it would be the next step to proceed. Towards the end, we were also able to record the CPMG experiments in the glycerol containing sample and I want to briefly contrast the curves for the fastest and slowest dynamic residues as mentioned in the above observations. Fig. 11 and 12 compare two residues from the above results. Among the faster-motion residues, Q2 shows twice as faster rate while N60 shows similar rate in comparison to the reference sample. E51 in the slower regime shows a step-like decay behaviour and hence a bad fitting while N61 dynamics seems to be quenched completely reporting a slower exchange rate. If the data for E51 is taken upto just 10 kHz for fitting (initial decay), we observe slower exchange rate similar to N61. This suggests the possibility that the peptide flip motion has slowed down in the presence of glycerol. The CPMG data recorded in glycerol shows a lot of noise and hence concrete conclusions can't be drawn just by comparison of the fitted rate constants. But

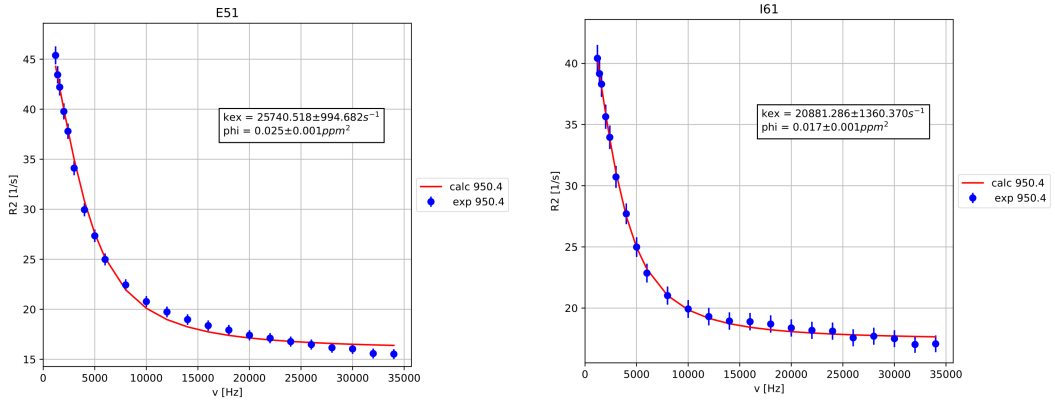

Figure 11: Two-state exchange fitting according to LM model for residues (E51 and I61) in the slowest exchange regime. Based on previous results from the lab, these residues most likely exhibit Peptide flip motion

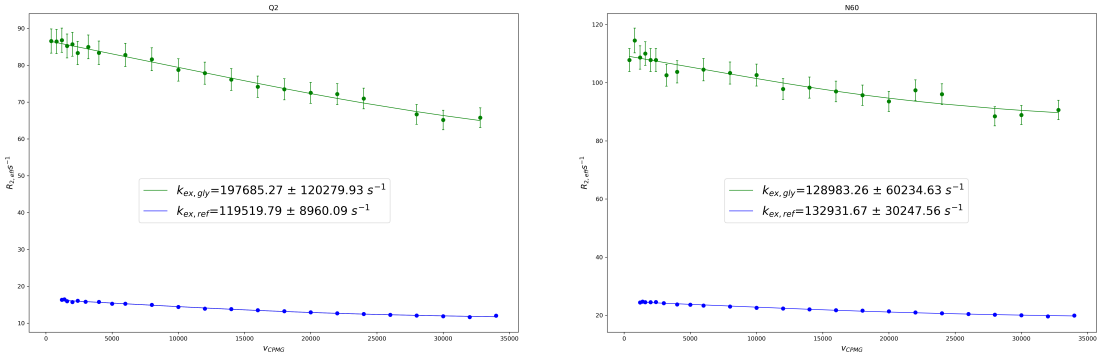

Figure 12: Comparison of two-state exchange fitting according to LM model for residues (Q2 and N60) in the fast-exchange regime from CPMG experiments in 40% glycerol (green) versus reference (blue)

the expectation that the motions we observe in the reference could be slowed down in the glycerol containing sample, does not appear to hold true for most of the residues. Also, the curves look more complicated and don't fit well according to a two-state model in the glycerol cases. But the slower-rate peptide flip motion appears to have slowed down in the presence of glycerol, based on the above fittings and disappearance of peak I23 (which otherwise strongly indicates peptide flip motion in the reference).

4 Discussion

Based on the results obtained so far, it has been difficult to clearly discern the ability of glycerol addition to help quantify motions in the μs time range. We were able to observe that glycerol addition increases τ_c but the CSP values indicate that the effect of glycerol addition might not be uniform across all residues and this could also possibly propagate and affect dynamics in the supra-tau_c range. As ^{15}N CPMG measurements

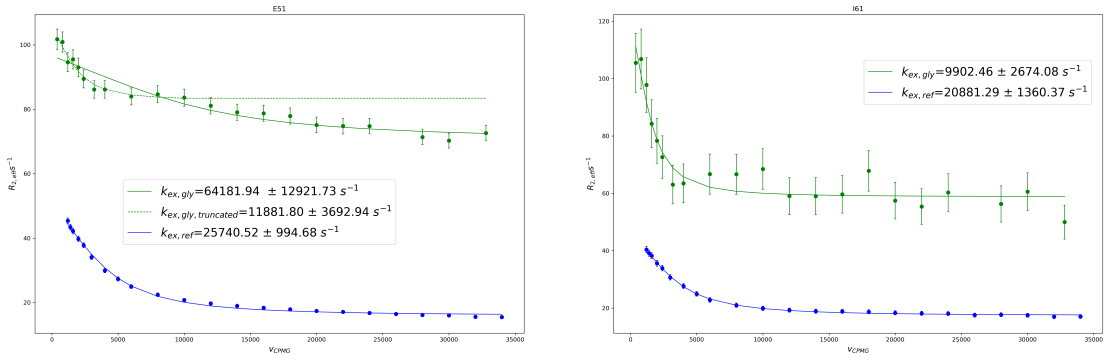

Figure 13: Comparison of two-state exchange fitting according to LM model for residues (E51 and N61) in the slow-exchange regime from CPMG experiments in 40% glycerol (green) versus reference (blue)

are very sensitive, random perturbations could easily obscure the results as well. One could possibly reperform the CPMG experiments at reduced glycerol concentration, or at higher temperature with the same concentration, to sort of regain the relaxation profiles as obtained from the reference sample.
From the R_1 and R_2 relaxation experiments at 277K, we calculated the global average τ_c and observed a 5-fold increase in the 40% glycerol samples as opposed to the reference. But since the rotational diffusion motion reflected by τ_c is most likely independent of motions in the μs-ms range, the effect of glycerol cannot be correlated directly.
From the CPMG experiments of ubiquitin in buffer at 277K, we saw two different scales of motion similar to what has been observed in the lab previously [12]. A Pincer mode motion most likely occurs in the time scale of $\sim 8\mu$s and a peptide flip motion $\sim 41\mu$s. Some residues experience exclusively either of the slow or fast dynamics, but most of the residues would be characterized by both ranges of motion. A double-LM fitting based on these two rates for the rest of the residues could be done as a starting point.
From the CPMG experiments of ubiquitin in 40% glycerol at 277K, I observed that the data shows a lot of noise, mostly because of low signal-to-noise ratio for some of the peak intensities and hence automatic model fitting doesn't apply very well. In the faster-regime many residues showed k_{ex} values higher than that recorded for glycerol in buffer but the errors were in the same order as the values and hence I couldn't make conclusions about the effect of glycerol in this regime. On the other hand, the residues showing slower dynamics reported lesser exchange rates than in the reference.

Hence, to conclude, glycerol addition slows down global tumbling and results in the increase of τ_c by a factor of 5 for ubiquitin at 277K. The role of glycerol in the faster regime of μs range dynamics (pincer mode motion) has been unclear due to the large uncertainties in the recorded data and the CPMG experiment needs to be repeated with different conditions. But in the slower-exchange regime (peptide flip motion), the dynamics of the involved residues appears to have slowed down by a factor of about 2.

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