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Applications of NMR-based PRE and EPR-based DEER Spectroscopy to Homodimer Chain Exchange Characterization and Structure Determination

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Abstract

The success of homodimer structure determination by conventional solution NMR spectroscopy relies greatly on interchain distance restraints (less than 6 Å) derived from nuclear Overhauser effects (NOEs) obtained from ¹³C-edited, ¹²C-filtered NOESY experiments. However, these experiments may fail when the mixed ¹³C-/¹²C-homodimer is never significantly populated due to slow homodimer chain exchange. Thus, knowledge of the homodimer chain exchange kinetics can be put to practical use in preparing samples using the traditional NMR method. Here, we described detailed procedures for using paramagnetic resonance enhancements (PREs) and EPR spectroscopy to measure homodimer chain exchange kinetics. In addition, PRE and EPR methods can be combined to provide mid-range (< 30 Å) and long-range (17- 80 Å) interchain distance restraints for homodimer structure determination as a supplement to short-range intrachain and interchain distance restraints (less than 6 Å) typically obtained from ¹H-¹H NOESY experiments. We present a summary of how to measure these distances using NMR-based PREs and EPR-based double electron electron resonance (DEER) measurements, and how to include them in homodimer structure calculations.

Keywords

NMR; EPR; spectroscopy; Homodimer; Chain exchange; Structure determination

1. Introduction

Structural genomics provides three dimensional (3D) structures of proteins on a genome wide scale by emphasizing high-throughput methods of structure determination. Owing to the numerous contributions from scientists working on the Protein Structure Initiative (PSI) project, the number of protein structures solved and deposited in the Protein Data Bank (PDB) increased dramatically in the past decade. Complete genome sequence information

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allows almost every open reading frame (ORF) to be cloned and expressed as a protein. The 3D structure of many of these proteins can then be determined at the atomic level using two main techniques: X-ray crystallography and Nuclear Magnetic Resonance (NMR) spectroscopy. In particular, the NMR method plays an important role for structure determination of proteins for which single crystals could not be obtained, making it a complementary method to X-ray crystallography (1). Protein NMR has become a routine method for structure determination for solution proteins with molecular mass under 25 kDa. However, structure determination of homodimer, oligomeric or protein-protein/DNA/RNA complexes is still challenging. Notwithstanding, the Northeast Structural Genomics (NESG) consortium has solved the structure of 26 oligomeric proteins out of 299 targets (306 pdb submissions) during the second phase of the PSI project (http://spine.nesg.org/nmr_progress.cgi). Given that about 80% of proteins from *Escherichia coli* have been predicted to exist as homo-oligomers based on a survey of their protein annotations in the Swiss-Prot database (2), and more than 86% of all proteins in the Swiss-Prot database were predicted to exist naturally as oligomeric complexes based on a computational prediction (3), more powerful and robust methods are needed for structural characterization of dimers or oligomers.

Prior to preparing the exchanged ^{13}C -/ ^{12}C -labeled sample to measure interchain nuclear Overhauser effects (NOEs) using the conventional NMR method (4–6) for homodimer structure determination, it is useful to have knowledge of the chain exchange kinetics. These can be obtained from techniques such as electronic paramagnetic resonance (EPR) spectroscopy and paramagnetic relaxation enhancement (PRE). In this chapter, we present protocols for making NMR-based PREs and EPR-based double electron electron resonance (DEER) measurements of homodimer chain exchange rate kinetics. The kinetics and thermodynamics of subunit exchange in multimeric proteins is of great potential significant to understanding functions in the cell that require exchanging binding partners in order to carry out specific biochemical or cellular functions (7,8).

In addition to traditional solution-state NMR spectroscopy using NOE-based methods, protein structure determination can integrate information obtained from other techniques such as EPR spectroscopy (9), PRE (9–11), residue dipolar coupling (RDC) (12) and small-angle X-ray scattering (SAXS) (13). In this chapter, we present a method for combining traditional NMR NOE restraints for homodimer structure determination with mid-range and long-range distance restraints using NMR-based PREs and EPR-based DEER measurements, respectively. The methods are demonstrated using the homodimer protein Dsy0195 from strictly anaerobic bacterium *Desulfitobacterium hafniense*. The details on the protein Dsy0195 constructs, expression, purification, and (*l*-oxy-2, 2, 5, 5-tetramethyl-*D*-pyrroline-3-methyl)-methanethiosulfonate (MTSL) spin labeling were previously published (9,14,15).

2. Materials

2.1 Equipment

In our laboratory, three NMR spectrometers were used for the structure determination studies and for measurement of chain exchange for homodimer Dsy0195: BrukerAvance III

850 MHz and 600 MHz spectrometers equipped with conventional 5 mm probes, and a Varian Inova 600 MHz with a 5 mm HCN cold probe (*see* Note 1). All DEER experiment data were collected on the spectrometer (Bruker ELEXSYS E580) with a SuperQft Q-band bridge and Q-band (EN 5107D2) probe (34 GHz), and analyzed by the software package DeerAnalysis2011 (requires Matlab) (16)

2.2 Protein samples

The locations of MTSL introduced into the homodimer protein of interest by site-direct spin labeling are critical to both PRE and DEER experiments. In this work, single cysteine mutants S36C and S52C of Dsy0195 were produced for DEER and PRE experiments (*see* Note 2). Protein samples of Dsy0195 prepared in this work included: wild type (*wt*) Dsy0195, ¹⁵N-Dsy0195, MTSL-Dsy0195-S52C, Dsy0195-S36C, and MTSL-Dsy0195-S36C. The protein concentrations were 1.0 mM for PRE experiments and 0.2 mM for DEER experiments, respectively (*see* Note 3).

2.3 Reagents and buffers

Prepare all solutions using 18 MΩ deionized water (LABCONCOPRO PS, Kansas city, MO, USA) and analytical grade reagents unless otherwise stated. Store all buffers and protein samples prepared at 4 °C before any EPR or NMR experiment.

1. 35 mM MTSL (Toronto Research Chemicals Inc.) in methanol
2. 150 mM ascorbic acid (Acros Organics)
3. PRE sample buffer: 20 mM ammonium acetate, 100 mM sodium chloride, 5 mM calcium chloride, 0.02 % (w/v) sodium azide, 50 μM DSS (for internal chemical shift referencing), 5% (v/v) D₂O, pH 4.5.
4. DEER sample buffer: 20 mM ammonium acetate, 100 mM sodium chloride, 5 mM calcium chloride, 0.02% (w/v) sodium azide, 30% (w/v) glycerol, pH 4.5 (*see* Note 4).

3. Methods

3.1 Chain exchange rate of Dsy0195 measured by DEER experiments

1. Dilute 0.2 mM MTSL-Dsy0195-S36C to 0.1 mM with the DEER buffer to generate the control sample. Transfer the control sample into an EPR capillary tube (*see* Note 5), and freeze carefully by dipping into liquid nitrogen. All DEER data were

¹Although we report the NMR spectrometers that were specifically used in our lab, any NMR spectrometer with 500 MHz or greater could be used for these experiments. Since our PRE experimental data were collected on a Bruker Avance III 600 MHz spectrometer, the parameter names given for the 2D SOFAST-HMQC in this chapter are consistent with the Bruker NMR software package TopSpin.

²Distances between protons of interest and the MTSL nitroxide that are within 30 Å can be derived by PRE experiments, whereas the measurable distances between two nitroxides range from 17 to 80 Å for DEER experiments. In this work, the distance between the C_β of S36 in the two chains is about 33.5 Å based on the crystal structure, which is suitable for DEER measurements, while the distance for S52 is about 15 Å, suitable for PRE experiments.

³In general, higher protein concentrations (~ 1 mM or greater) will provide the best signal-to-noise ratios and shorter data collection times for NMR and PRE experiments as long as protein does not aggregate. In contrast, protein concentrations of about 0.1 – 0.2 mM are optimal for the DEER experiment using a Q-band probe for good signal-to-noise with the benefit of avoiding the strong background signal of interchain molecular electron-electron interaction.

⁴Homogenous distributions of homodimers is achieved by addition of 30% glycerol as a cryoprotectant.

collected on a Bruker ELEXSYS E580 spectrometer with a SuperQft pulse bridge, 10 W amplifier and Q-band (EN 5107D2) probe at 80 K.

2. Find the resonator dip (*see* Note 6). In order to ensure that consistent levels of microwave power were achieved, a screen capture of the dip in the tuning window within the Bruker Xepir software was taken once the tuning was optimized for the first sample. For subsequent samples, the sample was manipulated within the resonator and the dip was compared to the screen capture until an identical frequency and coupling was obtained.
3. DEER experimental setup. The relaxation times T_1 and T_2 were measured to optimize the shot repetition time and DEER end point respectively. The pulse lengths were optimized to be as short as possible to yield a maximum modulation depth (*see* Note 7). In this case, 10 ns ($\pi/2$) and 20 ns (π) probe pulses and a 24 ns (π) pump pulse were used; however, this is spectrometer dependent and will have to be determined in each case. The data were collected with an 80 MHz frequency separation between the pump and probe frequencies. Collect the DEER data.
4. Repeat the DEER experiment of the control sample four more times to obtain the measurement reproducibility. Fig. 1A shows the background subtracted and scaled time domain data for 5 repeated DEER measurements of the control sample (*see* Note 8). The modulation depth is measured as the percent difference between the maximum intensity at time zero and the first minimum, which in this case was 14%. The repeated results shown in Fig. 1A demonstrate the small experimental deviation (Fig. 1A).
5. Mix 10 μ l of 0.2 mM Dsy0195-S36C and 10 μ l of 0.2 mM MTSL-Dsy0195-S36C, transfer into EPR tube, and freeze in liquid nitrogen at a dimer exchange time of approximately one hour to attempt to estimate the exchange rate. Collect the DEER data as in **step 3**. Based on the obtained modulation depth from this experiment, determine a range of appropriate time points for the remaining experiments.
6. Repeat **step 5** with a series of exchange time times as determined in the previous step, in this case, 2.5, 5, 10, 15, 25, 35, 52.5, 112.5, and 155 minutes (*see* Note 9).

⁵ For these measurements, it is critical that the sample height within the tube is kept consistent so that identical levels of tuning can be obtained from sample to sample.

⁶ As the primary objective of these measurements is the precise determination of the modulation depth in the DEER experiment, achieving consistent coupling is essential. In a typical Q-band setup, the coupling is less overcoupled than in an X-band experiment. As such, the pump and probe frequency separation is on the order of the width of the resonator bandwidth, making the microwave power achieved at the two frequencies very sensitive to the tuning.

⁷ In a typical DEER experiment, one collects data to as long an end point as possible given the limitation of the T_2 of the sample. This yields the most precise distance distribution information. In these exchange experiments, since it is only the modulation depth which is being determined, one can shorten the end point of the experiment to enhance the signal to noise and sample throughput as a precise distance determination is not the primary goal.

⁸ Quantitative determination of the modulation depth from sample to sample depends on consistent analysis of the data, particularly the subtraction of the intermolecular background signal. This was accomplished using the DEERAnalysis2011 package from the Jeshcke laboratory (16). A detailed description of the analysis is out of the scope of this chapter, but the included documentation with the software is extensive.

⁹ These selected incubation times for chain exchange were appropriate for the system studied, but these can be adjusted based on the exchange rate of each individual system. The first data point is limited to the time it takes to mix the MTSL labeled and unlabeled protein, load the tube and freeze the sample in liquid nitrogen. In practice, this could potentially be as low as a one minute given conventional methods. If shorter times are needed in the case of very rapid dimer exchange rates, rapid freeze quench (RFQ) methods could potentially be adapted.

7. The scaled modulation depth for each measurement was derived as in **step 4**. All modulation depths were scaled to the value of the control sample, in which the depth of 0.14 was scaled to 1.
8. Plot the curve of the normalized modulation depth versus the exchange time. Curve fitting obeys a first-order exponential decay (Fig. 1C), and the homodimer chain exchange rate of 0.039 min^{-1} is derived from the fit.

3.2 Chain exchange rate of Dsy0195 measured by PRE experiments (see Note 10)

1. Prepare the control sample without chain exchange. Dilute 150 μl of 1.0 mM ^{15}N -Dsy0195 with the PRE buffer to a final concentration of 0.5 mM as the control sample. Transfer the control sample into the NMR tube and put into the NMR spectrometer.
2. Setup the NMR acquisition parameters. Load the standard 2D SOFAST-HMQC pulse sequence `sfhmqcf3gpqh`, and set pulse sequence parameters by default values including the flip angle for the first proton pulse of 1200° , the band-selective proton pulse centered at 8.0 ppm covering a bandwidth of 4.0 ppm. The acquisition parameters were setup as follows: spectrum width, $\text{SW}_1 \times \text{SW}_2 = 29 \times 15 \text{ ppm}$ for ^{15}N and ^1H (optimized from the chemical shift dispersions in both dimension); time domain data set, $\text{TD}_1 \times \text{TD}_2 = 80 \times 1,024$; number of scan, $\text{ns} = 4$; acquisition time for the ^1H dimension of 47ms, and the maximum acquisition time for the ^{15}N dimension of 23 ms; recycle time, $\text{d1} = 100 \text{ ms}$. The total experimental time is 58 seconds. Collect the 2D SOFAST-HMQC spectrum.
3. Process the NMR data with NMRPipe (17). The time domain data were Fourier transformed in both dimensions after zero fill in the $t_1 \times t_2$ dimensions to $128 \times 2,048$ points, applying a window function of a 90° shifted sine-bell and mirror image linear prediction (LP) in the ^{15}N dimension. The processed spectra were visualized using SPARKY(18) and peak intensities were obtained from SPARKY peak heights.
4. Peak intensity normalization. The cross peak intensities were normalized with the average intensity from the seven backbone peaks K44, E41, G55, E56, K57, L58 and G59, for which distances between amide protons of interest in one chain to the C_β of S52 in the other chain are greater than $30 \text{ \AA}$ based on the crystal structure of Dsy0195 (see Note 11).
5. Estimate the peak intensity deviations of control sample. Repeat the data collection four times at an interval time of approximately ten minutes. Fig. 2A showed the

¹⁰The mixture of ^{15}N -labeled Dsy0195 and MTSL-labeled Dsy0195-S52C in 1:1 ratio were prepared (the ratio can be different) (15), and fast NMR data collection using 2D SOFAST-HMQC (26) experiments were carried out at a series of mixing times for dimer chain exchange. Samples were put into 5 mm Shigemi NMR tubes with magnetic susceptibility matched to solvent D_2O (BMS-003, Shigemi, Inc., Allison Park, PA, USA). All spectra were carried out on a Bruker Avance III 600 MHz spectrometer at 293 K.

¹¹In the NMR spectra of mixed samples, NMR signals are coming from two dimer forms: ^{15}N -Dsy0195, and ^{15}N -Dsy0195/MTSL-Dsy0195-S52C that was created by the chain exchange during the mixing time. PREs can only be observed for those amide protons in the ^{15}N -labeled chain of dimer ^{15}N -Dsy0195/MTSL-Dsy0195-S52C that are less than $30 \text{ \AA}$ away from the nitroxide in the other chain. For the other amide protons without PREs, the peak intensities in 2D HMQC will remain unchanged during the chain exchange process. These are candidates for normalization of peaks in the entire spectrum.

average peak intensity and standard deviation versus individual amino acid (39 backbone peaks without overlap). The plot indicated the errors in peak intensity reproducibility could be up to 15%.

6. Mixed sample preparation and NMR data collection. Mix 150 μ l of 1.0 mM U - ^{15}N -Dsy0195 and 150 μ l of 1.0 mM MTSL-Dsy0195-S52C. Transfer the mixed sample into NMR tube, put into NMR magnet. Lock the signal, tune and match the probe, shim the field, determine the solvent carrier frequency and 90° pulse width as quickly as possible (can be done within 5 min). Collect the spectra at a series of chain exchange times 6, 8, 10, 15, 20, 25, 35, 45, 52.5, 60, 70, 90, 112.5, 140, 155, 180 and 210 min (*see* Note 12).
7. Process the data and normalize the peak intensities as in **steps 3** and **4**.
8. Repeat **steps 6** and **7** four more times. The average peak intensity and standard deviation for each peak can be obtained from the five repeated measurements.
9. Derive the homodimer chain exchange rate. Plot the peak intensity versus exchange time for each residue that had a decrease in peak intensity. This curve for residue E84 is given as an example (Figure 2B). The curve fitting followed the first-order exponential decay, and indicated that the chain exchange rate of Dsy0195 was $0.037 \pm 0.008 \text{ min}^{-1}$ (*see* Note 13).

3.3 Structure determination of homodimer Dsy0195 by combining NMR and EPR derived interchain distance restraints

3.3.1 Overview—The initial Dsy0195 monomer structure was determined following the conventional protocols of our laboratory, referred to in recent structure publications (19–22). The monomer structure quality was assessed using software package PSVS 1.4 (23). Although the overall tertiary structure was correct, many distance violations were present in both N-terminus and C-terminus of Dsy0195 that are located at the homodimer interface known from its crystal structure. These violations could result from some interchain NOEs were automatically assigned as interchain NOEs during structure calculations using CYANA (24) with input of peak lists of NOESY spectra. An additional problem in the structure determination of the homodimer Dsy0195 by NMR spectroscopy was that few interchain-NOEs were detected from the ^{13}C -edited, ^{12}C -filtered NOESY experiment, which led to either poor quality of the homodimer structure solved by NMR method alone (14). These problems were overcome by using the mid-range and long-range interchain distance restraints that were derived from PRE and DEER experiments (9,14). After these interchain distance restraints were used as input, more interchain NOEs were assigned in the conventional ^{13}C -edited NOESY spectra by the CYANA program (9). In this chapter, we focus on deriving these mid-range and long-range distance restraints by PRE and DEER experiments and using them in the structure determination of homodimer Dsy0195 along with conventional NMR data using the CYANA program.

¹²The optimal exchange times will vary significantly for each dimer depending on the chain exchange rate.

¹³Although the plot of peak intensity versus chain exchange time of each individual peak can be plotted, the successful curve fitting following the first exponential decay is not guaranteed. Especially, for the residues with no PREs or weak PREs, the curve fitting will not follow a first-order exponential decay due to no or little change of the peak intensity.

3.3.2 Long-range distance restraints derived from DEER experiments

1. Sample preparation. Two samples each of 0.2 mM MTSL-Dsy0195-S36C and MTSL-Dsy0195-S52C were prepared for DEER experiments.
2. Collect the DEER data as in in Subheading 3.1 **step 3**. Distances were derived using the DEERAnalysis2011 package from the Jeschke laboratory at ETH using a Tikhonov regularization. As mentioned above, this software is well documented and a detailed description of the analysis is out of the scope of this chapter.
3. Two distances of 34 and 20 Å between two nitroxides in the MTSL-labeled homodimers S36C and S52C were derived, respectively. Considering that the free radical of the nitroxide is typical 8 – 10 Å from its own backbone amide, the error bar for DEER distance restraints is ± 5 Å in the following structure calculation.

3.3.3 Mid-range distance restraints derived from PRE experiments

1. Mixed sample with PREs (referred to as paramagnetic state, or reduced state) preparation. Mix 150 μ l of 1.0 mM ^{15}N -labeled Dsy0195 and 150 μ l of 1.0 mM MTSL-Dsy0195-S52C. Transfer the mixture into the NMR tube and put into the NMR spectrometer. The experiment was carried out at 293 K.
2. NMR data collection. Collect 2D ^1H - ^{15}N HSQC in the reduced state (*see* Note 14). After the experiment, add about 3 – 4 μ l 150 mM ascorbic acid (correspondingly 2–3 fold molar excess to MTSL) to get rid of MTSL from the protein (referred to as diamagnetic state or oxidized state). Collect the 2D ^1H - ^{15}N HSQC in the diamagnetic state with the same parameters as the paramagnetic state.
3. Process the NMR data with NMRPipe. The time domain data were Fourier transformed in both dimensions after zero fill at $t_1 \times t_2$ dimensions of 512 x 2048 and application of a 90° shifted sine-bell window function. The processed spectra were visualized and peak picked using SPARKY.
4. Peak intensity normalization. Measure the intensity of each resonance from the spectrum of mixed sample before the addition of ascorbic acid, recorded as $I_{\text{para}*}$, that of the mixed sample after the addition of ascorbic acid, recorded as I_{dia} . Peak intensities were normalized as the same in Subheading 3.2 **step 4**. Then, the peak intensity of solely paramagnetic state I_{para} is equal to $2(I_{\text{para}*} - I_{\text{dia}}/2)$ (equation 3 in reference (11)), where I_{dia} is the intensity for diamagnetic state (*see* Note 15).
5. Mid-range interchain distance restraints. Based on the ratio of peak intensity ($I_{\text{para}}/I_{\text{dia}}$) for each resonance, the interchain distances were derived following the equations (4) and (5) in the literature (11). Based on the calculation, the distances for peak intensity ratios of 0.9 and 0.1 are about 22 and 13 Å, respectively. Three classes of distance restraints were used for structure calculation. Peaks with an

¹⁴The acquisition parameters are setup as follows: spectrum width: $\text{SW}_1 \times \text{SW}_2 = 29 \times 18$ ppm (optimized from the chemical shift dispersions in both dimension), time domain data set, $\text{TD}_1 \times \text{TD}_2 = 256 \times 1,024$; acquisition time (AQ) at ^1H dimensions of 34ms and the maximum acquisition at ^{15}N dimension of 51 ms; number of scan, NS = 128; recycle time, d1 = 1 s. The total experimental time is about 10 hours.

¹⁵The intensity of each peak ($I_{\text{para}*} - I_{\text{dia}}/2$) in paramagnetic state comes only from the dimer ^{15}N - Dsy0195/MTSL-Dsy0195-S52C, which accounts for 50% of the sample in the mixture of 1:1 ^{15}N -Dsy0195 and MTSL-Dsy0195-S52C (11).

intensity ratio > 0.9 , were restrained only with a lower bound of 22 Å; peaks with an intensity ratio < 0.1 , were restrained only with an upper bound of 13 Å; peaks with intensity between 0.1 and 0.9, were restrained with ± 4 Å for upper and lower bound, respectively, for structure calculation. For Dsy0195, 67 lower-bound and 35 upper-bound interchain restraints were derived from PRE data.

3.3.4 Interchain NOEs determination and homodimer structure calculation—As additional input, the interchain restraints from both PRE and DEER experiments were in the structure calculation. We used the CYANA program to calculate the homodimer structure with the chemical shift assignments, NOESY peaklists, dihedral-angle restraints, backbone hydrogen-bond restraints. The homodimer structure calculation resulted in a converged ensemble of structures. We assessed the structure quality using PSVS 1.4, and checked the NOE assignments manually based on the NOE and dihedral-angle violations.

3.3.5 Homodimer structure refinement (see Note 16)—Convert the final NOE-derived distance restraints from CYANA, dihedral-angle restraints, and hydrogen-bond restraints to Xplor/CNS format using PDBStat (23). These restraints were used to calculate 100 structures using Xplor-NIH followed by refinement of the 20 lowest energy structure using restrained molecular dynamics in explicit water with CNS 1.2 (CNSw) (25). Use PSVS 1.4 to assess the structure quality and check the restraints violation. The final NMR ensemble of 20 structures with the lowest energy was deposited in the PDB (PDB ID 2KYI).

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¹⁶This step was done following our laboratory protocols at the time when the structure of Dsy0195 was determined. The protocols of protein structure calculation and refinement are different from lab to lab.

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Fig. 1. Homodimer chain exchange rate measurement using DEER experiment. (A) Overlay of scaled refocused echo intensity versus phase memory time for the control sample (repeated five times). (B) Time-domain DEER signals of 1:1 mixture of Dsy0195-S36C-MTSL/¹⁵N-Dsy0195-S36C at a series of exchange time points: 0, 2.5, 5, 10, 15, 25, 35, 52.5, 70, 112.5, 155 minutes. (C) Plot of the normalized modulation depth versus chain exchange time following the first-order exponential decay. The blue filled circle stands for the experimental depth and red solid line for the fitting curve following the first-order exponential decay.

Fig. 2.

Homodimer chain exchange rate measurement using PRE experiment. (A) Plot of the normalized peak intensity (average from five repeats) of 39 backbone amide peaks without overlap in 2D SOFAST-HMQC spectra of ^{15}N -Dsy0195. The blue circle stands for the average intensity, and the pink line for the standard deviation. (B) Plot of the normalized intensity (average from five repeats) versus chain exchange time for a given residue (E84) as an example. The blue filled circle stands for the peak intensity, pink line for standard deviation, and the red solid line for the fitting curve following the first-order exponential decay.