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Mapping Protein-Protein Interactions by Double-REDOR-Filtered Magic Angle Spinning NMR Spectroscopy

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Abstract

REDOR-based experiments with simultaneous ^1H - ^{13}C and ^1H - ^{15}N dipolar dephasing are explored for investigating intermolecular protein-protein interfaces in complexes formed by a $\text{U-}^{13}\text{C}$, ^{15}N -labeled protein and its natural abundance binding partner. The application of a double-REDOR filter (dREDOR) results in a complete dephasing of proton magnetization in the $\text{U-}^{13}\text{C}$, ^{15}N -enriched molecule while the proton magnetization of the unlabeled binding partner is not dephased. This retained proton magnetization is then transferred across the intermolecular interface by ^1H - ^{13}C or ^1H - ^{15}N cross polarization, permitting to establish the residues of the $\text{U-}^{13}\text{C}$, ^{15}N -labeled protein, which constitute the binding interface. To assign the interface residues, this dREDOR-CPMAS element is incorporated as a building block into ^{13}C - ^{13}C correlation experiments. We established the validity of this approach on $\text{U-}^{13}\text{C}$, ^{15}N -Histidine and on a structurally characterized complex of dynactin's $\text{U-}^{13}\text{C}$, ^{15}N -CAP-Gly domain with end-binding protein 1 (EB1). The approach introduced here is broadly applicable to the analysis of intermolecular interfaces when one of the binding partners in a complex cannot be isotopically labeled.

INTRODUCTION

Magic angle spinning (MAS) NMR is a powerful method for atomic-resolution studies of structure and dynamics of proteins and protein assemblies. For insoluble and non-crystalline macromolecular systems, such as complexes of cytoskeleton-associated (Shi et al. 2015; Vasa et al. 2015; Yan et al. 2013a) and membrane proteins (Cady et al. 2010; Park et al. 2012), fibrillar aggregates (Colvin et al. 2016; Hoop et al. 2016) and viral assemblies (Andreas et al. 2016; Morag et al. 2014; Morag et al. 2015; Sborgi et al. 2015), MAS NMR is often the only technique yielding atomic-resolution structural information.

Biological functions of proteins typically require their association with other proteins or ligands (Phizicky and Fields 1995; Vale 2003) and, therefore, the understanding of the functionally important protein-protein interactions at atomic level is essential. MAS NMR is commonly employed for the investigation of protein-protein and protein-ligand interactions, and two main approaches are typically followed. One is to explore the effect of binding or

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complex formation on the conformational changes by comparing the chemical shifts or peak intensities before and after binding (Asami et al. 2013). This approach has been applied to probe intermolecular protein or ligand-binding interfaces in a variety of protein complexes in the solid state, e.g. Bcl-xL (Zech et al. 2004), and Congo red/amyloid (Schutz et al. 2011). In these examples structural perturbations and differential binding sites between ligands were monitored by chemical shift changes. Another approach is to acquire cross-interface heteronuclear correlations through dipolar couplings in differentially labeled samples (Weingarth and Baldus 2013; Yan et al. 2013b; Yang et al. 2008). The first approach yields information about perturbations of the structure both due to direct interactions and to allosteric effects. The second approach requires either that both binding partners are isotopically labeled or that one is labeled with ^2H , ^{13}C , and ^{15}N while the other is unlabeled.

We have previously introduced various types of REDOR filters for studies of intermolecular interfaces in differentially labeled protein assemblies, using as a model system the 1–73($\text{U-}^{13}\text{C},^{15}\text{N}$)/74–108($\text{U-}^{15}\text{N}$)-thioredoxin reassembly (Yang et al. 2008). For example, in one of these experiments dubbed REDOR-PAINCP, the ^{15}N magnetization from 1–73- $\text{U-}^{13}\text{C},^{15}\text{N}$ -enriched fragment was dephased by the $^{15}\text{N-}^{13}\text{C}$ REDOR filter, and the remaining magnetization from the 74–108($\text{U-}^{15}\text{N}$)-fragment was transferred across the intermolecular interface to the ^{13}C atoms of the 1–73- $\text{U-}^{13}\text{C},^{15}\text{N}$ -fragment. It was demonstrated in that study that, depending on the implementation of different filters, the dephasing profiles give rise to different kinds of cross peaks in the resulting 2D REDOR-filtered spectra. REDOR-HETCOR, HETCOR-REDOR, and PDS-REDOR experiments were also introduced in that study. This approach allows for resonance assignments of two binding partners as well as for structural analysis of the intermolecular interfaces, and is applicable to ($\text{U-}^{13}\text{C},^{15}\text{N}/\text{U-}^{15}\text{N}$) or ($\text{U-}^{13}\text{C},^{15}\text{N}/\text{U-}^2\text{H},^{15}\text{N}$) differentially enriched protein complexes. However, these experiments require that both binding partners are isotopically labeled and hence cannot be performed on complexes where only one molecule contains labels. There are a number of biomolecular systems where currently isotopic labeling is not possible, with cytoskeleton filaments (microtubules and actin) being one such example. The dREDOR method discussed here fills this gap and allows for structural analysis of interfaces formed by a pair of proteins where only one partner is labeled.

As we have demonstrated recently in the studies of $\text{U-}^{13}\text{C},^{15}\text{N}$ -CAP-Gly domain of mammalian dynactin assembled with polymeric microtubules, the first method reports on both the direct interactions and allosteric structural perturbations while the second one is not applicable to CAP-Gly/microtubule assemblies as isotopic labeling of microtubules cannot be readily performed at this time (Wall et al. 2016). We have therefore explored an alternative approach for gaining atomic-resolution information into the interfaces formed by $\text{U-}^{13}\text{C},^{15}\text{N}$ -CAP-Gly and natural abundance microtubules, dubbed dREDOR. The experiment starts by the application of a simultaneous $^1\text{H-}^{13}\text{C}$ and $^1\text{H-}^{15}\text{N}$ REDOR (double REDOR, dREDOR) filters of various durations to the complexes of $\text{U-}^{13}\text{C},^{15}\text{N}$ labeled protein with its unlabeled binding partner, followed by $^1\text{H-}^{13}\text{C}$ or $^1\text{H-}^{15}\text{N}$ cross polarization transfer and, if desired, the subsequent $^{13}\text{C-}^{13}\text{C}$, $^{15}\text{N-}^{13}\text{C}$ or $^{13}\text{C-}^1\text{H}$ mixing periods (Yan et al. 2015a). The dREDOR filter in the first step dephases the magnetization of the protons that are directly bonded to ^{13}C and ^{15}N labeled sites (i.e., all protons in the $\text{U-}^{13}\text{C},^{15}\text{N}$ labeled protein). The remaining proton magnetization, which originates from the unlabeled

molecule, is then transferred across the intermolecular interface back to the labeled molecule, during the ^1H - ^{13}C or ^1H - ^{15}N cross polarization (CP) step. Finally, the residues situated at the interface can be assigned by employing a subsequent intramolecular homonuclear or heteronuclear correlation step.

In this work, we present an in-depth analysis of the dREDOR based experiments. We established the dREDOR dephasing dynamics in a series of 1D dREDOR-CPMAS experiments on two control samples, $\text{U-}^{13}\text{C}$, ^{15}N -Histidine and $\text{U-}^{13}\text{C}$, ^{15}N -dynein's light chain 8 protein, LC8. The effects of dREDOR on quasi-uniform dipolar dephasing during the filter time in both samples are discussed. We then applied dREDOR to probe the intermolecular binding interface formed by $\text{U-}^{13}\text{C}$, ^{15}N -CAP-Gly in complex with unlabeled end-binding 1 protein (EB1). Our results establish the general requirements for dREDOR based experiments that are applicable to a wide variety of protein complexes.

MATERIALS AND METHODS

Sample preparation

$\text{U-}^{13}\text{C}$, ^{15}N -labeled L-histidine was purchased from Cambridge Isotope Laboratories and recrystallized at pH 6.0. The sample contains histidine crystallites of two protonation states: the biprotonated state (+1) and neutral τ tautomer state (τ). The $\text{U-}^{13}\text{C}$, ^{15}N -labeled LC8 sample containing 5 mM Cu(II)-EDTA was prepared as described previously.(Sun et al. 2012) The LC8 protein precipitated from PEG was packed into a 1.9 mm Bruker MAS rotor. The $\text{U-}^{13}\text{C}$, ^{15}N CAP-Gly sample was prepared by controlled precipitation from polyethylene glycol (PEG) as described previously (Marulanda et al. 2004; Sun et al. 2009), and 24.4 mg of the precipitate were packed into a 3.2 mm Bruker rotor. $\text{U-}^{13}\text{C}$, ^{15}N CAP-Gly in complex with natural abundance EB1 was prepared at the molar ratio of 1:1 as described previously (Yan et al. 2013a), and 23.5 mg of hydrated solid-state NMR sample were packed into a 3.2 mm Bruker rotor.

MAS NMR spectroscopy

The MAS NMR spectra of $\text{U-}^{13}\text{C}$, ^{15}N -histidine and $\text{U-}^{13}\text{C}$, ^{15}N -LC8 were acquired on a Bruker AVIII spectrometer (19.96 T) using a 1.9 mm triple-resonance MAS probe. The Larmor frequencies were 850.4 MHz (^1H), 213.8 MHz (^{13}C), and 86.2 MHz (^{15}N). The spectra of free $\text{U-}^{13}\text{C}$, ^{15}N -CAP-Gly and 2D dREDOR-filtered CORD (Hou et al. 2013) spectrum of $\text{U-}^{13}\text{C}$, ^{15}N -CAP-Gly/EB1 complex were acquired on a 11.7 T Bruker AVIII spectrometer equipped with a 3.2 mm EFree HCN probe. The Larmor frequencies were 500.1 MHz (^1H), 125.8 MHz (^{13}C), and 50.7 MHz (^{15}N). The MAS frequency was set to 20 kHz for histidine and to 14 kHz for the other samples, and controlled to within ± 10 Hz by a Bruker MAS controller. The apparent temperature was maintained within ± 0.1 °C and the actual sample temperature was calibrated for this probe by KBr temperature sensor. The actual sample temperature was 20 °C for histidine, -16 °C for LC8 and free CAP-Gly, and -1 °C for CAP-Gly/EB1 complex. The 2D DARR spectrum of $\text{U-}^{13}\text{C}$, ^{15}N -CAP-Gly/EB1 complex was acquired at 19.96 T at +4 °C.

In the 1D double-REDOR (dREDOR) filtered experiments, the ^1H - ^{13}C and ^1H - ^{15}N REDOR pulses (Gullion and Schaefer 1989) are simultaneously applied after the ^1H excitation, followed by either acquisition on ^1H channel or Hartmann-Hahn cross polarization (CP) transfer to ^{13}C or ^{15}N nucleus for $^{13}\text{C}/^{15}\text{N}$ detection (Fig. 1). XY-8 phasing scheme was applied during the rotor-synchronized REDOR π -pulse train. For the 19.96 T NMR experiments carried out on histidine and LC8, each data set contains the following four spectra: 1) with ^1H evolution and without REDOR pulses on (S_0); 2) with ^1H - ^{13}C REDOR pulse; 3) with ^1H - ^{15}N REDOR pulse; 4) with double ^1H - $^{13}\text{C}/^1\text{H}$ - ^{15}N REDOR pulses. The REDOR pulses are incremented by 2 rotor periods ($2\tau_r$), and the total dephasing time in these experiments ranges from $2\tau_r$ to $50\tau_r$. The number of scans was increased for long REDOR durations to yield sufficient sensitivity. The typical 90° pulse lengths were $2.4\ \mu\text{s}$ for ^1H , $2.9\ \mu\text{s}$ for ^{13}C and $3.2\ \mu\text{s}$ for ^{15}N . The contact time for both (^{13}C)CPMAS and (^{15}N)CPMAS was 1 ms. For LC8, an extra set of spectra were collected with a pre-saturation $\pi/2$ -pulse train applied on ^{13}C channel before the proton excitation, to avoid residual signals from a previous scan. The 1D dREDOR-based experiments recorded in this work are without pre-saturation except where specified.

For free CAP-Gly and CAP-Gly/EB1 complex, ^1H - ^{13}C REDOR-CPMAS and dREDOR-CPMAS spectra were collected at 11.7 T with various REDOR duration times. The 90° pulse lengths were $2.7\ \mu\text{s}$ for ^1H , $3.2\ \mu\text{s}$ for ^{13}C , and $5.0\ \mu\text{s}$ for ^{15}N . Data sets were acquired with CP contact times of 5 and 1 ms. The 2D dREDOR-CORD experiment was recorded with the CORD mixing time of 50 ms, and a CP contact time of 5 ms. During the t_1 and t_2 evolution, the SPINAL-64 (Fung et al. 2000) decoupling pulse was applied on ^1H channel.

Processing and analysis of NMR spectra

All 1D REDOR-based spectra were processed and analyzed in MNova NMR. The peak intensities were extracted from the ^1H , ^{13}C and ^{15}N spectra of U- ^{13}C , ^{15}N -labeled histidine and used for calculating the ratio of reduced REDOR signal and full signal S/S_0 . For U- ^{13}C , ^{15}N -LC8, U- ^{13}C , ^{15}N -CAP-Gly, and U- ^{13}C , ^{15}N -CAP-Gly/EB1, the integrals corresponding to different chemical shift regions were extracted and used in the analysis, since individual peaks are not well resolved in the corresponding 1D spectra. In the ^{13}C spectra the regions corresponding to aliphatic sidechains, C^α backbone, aromatic sidechains, and carbonyls are 5–45, 45–71, 100–140, and 160–190 ppm, respectively. The REDOR and dREDOR dephasing curves were fitted to an exponential decay function, and the dephasing rates were quantitated by the respective time constants (see Table 1 and Table 2). The 2D dREDOR-CORD spectrum was processed in NMRPipe (Delaglio et al. 1995) and analyzed with Sparky (Goddard and Kneller 2008).

RESULTS

Efficiency of single vs. double REDOR filters in rigid environments: histidine

The 1D ^1H , ^1H - ^{13}C CPMAS and ^1H - ^{15}N CPMAS spectra of U- ^{13}C , ^{15}N -histidine with and without the double-REDOR filter are shown in Fig. 2. In this sample, two charged states co-exist, the biprotonated state (noted as “+1”, ball and stick representations are shown in Fig. 2E) and neutral τ tautomer state (noted as “ τ ”) (Li and Hong 2011). According to the peak

intensities, the ratio of two forms is approximately 3:1. Since the histidine molecule is rigid, all signals in the ^{13}C and ^{15}N CPMAS spectra are suppressed with a dREDOR dephasing time of 700 μs ($14\tau_{\text{R}}$, $\nu_{\text{R}}=20$ kHz). Several signals have peak intensities that are distinguishable from the noise level with 800 μs dREDOR dephasing (e.g., for the C' , C_{α} , C_{β} and $\text{H}_{\text{e}2}$ atoms), but their intensities are less than 2% of the full intensities in the regular CPMAS spectra. Thus these residual signals are negligible and their corresponding resonances are considered to be nearly 100% suppressed by the dREDOR dephasing. We note that a dREDOR dephasing time of 500 μs is already long enough to remove the majority magnetization of CH or NH protons, as can be concluded from the S/S_0 dephasing curves of each nucleus.

The experimental REDOR dephasing curves of ^1H , ^{13}C and ^{15}N spins in U- ^{13}C , ^{15}N -histidine are extracted from the REDOR/dREDOR-based ^1H , ^{13}C and ^{15}N CPMAS spectra, and are shown in Fig. 2, Fig. S1 and Fig. S2, respectively. The signal intensity fractions S/S_0 are shown as function of the REDOR dephasing time. Considering the complexity in terms of size and orientation of dipolar couplings in multiple spin pairs in histidine and protein systems, we have used a single exponential function to approximate the dipolar dephasing processes and analyze the decay rates quasi-quantitatively:

$$y = y_0 + A\exp(-x/\tau)$$

where $1/\tau$ denotes the exponential decay rate constant, which is related to the efficiency of the respective dipolar filter (H-C REDOR, H-N REDOR, or dREDOR). The fitted dephasing curves for each REDOR or dREDOR filter are shown in Fig. 2. The corresponding REDOR dephasing rates for each spin in histidine are summarized in Table 1.

Dephasing curves of the single ^1H - ^{13}C and ^1H - ^{15}N REDOR filters are compared with that when dREDOR filter was used. As shown in Fig. 2 A-D, the efficiency of ^1H - ^{13}C REDOR experiment is similar to the dREDOR sequence for dephasing the ^{13}C -bound protons but significantly lower for dephasing the ^{15}N -bound protons. In the ^{13}C -detected experiments, the ^1H - ^{15}N REDOR is much less efficient than both ^1H - ^{13}C REDOR and dREDOR when dephasing protons that are close to the ^{13}C site. This is expected since most of the ^1H - ^{15}N distances in histidine are medium-range (two- or three- bonds) except for the three direct-bond ^1H - ^{15}N spin pairs ($^1\text{H}_{\alpha}$ - $^{15}\text{N}_{\alpha}$, $^1\text{H}_{\delta}$ - $^{15}\text{N}_{\delta}$, $^1\text{H}_{\text{e}2}$ - $^{15}\text{N}_{\text{e}2}$). In the ^{15}N -detected experiments, ^1H - ^{15}N REDOR is more efficient than ^1H - ^{13}C REDOR except for the $^{15}\text{N}_{\delta}$ (τ tautomer) site. The latter has a slowly decaying ^1H - ^{15}N REDOR curve since it does not have directly bonded ^1H and hence the ^1H - $^{15}\text{N}_{\delta}$ dipolar couplings are weak. These results illustrate that neither of the two conventional single REDOR schemes can filter out the dipole-dipole coupled spins as efficiently as dREDOR. We also note that dephasing curves for $^1\text{H}_{\delta 1}$ and $^1\text{H}_{\text{e}2}$ atoms were derived separately based on the one-pulse ^1H spectrum, but other proton signals are not well resolved, and their dephasing profiles are hence not available from this work.

Numerical simulations for an isolated spin pair: dREDOR dephasing time for rigid and mobile environments

Fig. 3 shows the simulated ^1H - ^{13}C REDOR, ^1H - ^{15}N REDOR and dREDOR dephasing curves of ^{13}C - or ^{15}N -bonded protons in an isolated ^1H - ^{13}C - ^{15}N - ^1H spin system. The numerical simulation is performed using SIMPSON (Bak et al. 2000). The values of ^1H - ^{13}C and ^1H - ^{15}N heteronuclear dipolar couplings as well as ^1H - ^1H homonuclear dipolar couplings are calculated for the proton marked in red dashed circle and are taken as inputs for the simulations in three models: rigid-molecule, mobile-molecule and partially-mobile-molecule. In the mobile model the dipolar order parameter of 0.2 (1/5 of the order parameter of the rigid bond) was assumed for both ^1H - ^{13}C and ^1H - ^{15}N dipolar couplings. In the partially mobile model the dipolar order parameter for ^1H - ^{13}C bond was set to 0.2 while other bonds were assumed to be rigid with order parameter of 1.0. For all three models, the simulations indicate that the effect of dREDOR dephasing is the same as the effect of ^1H - ^{13}C REDOR for the ^{13}C -bonded proton and similar to that of the ^1H - ^{15}N REDOR for the ^{15}N -bonded proton. However, a single ^1H - ^{13}C REDOR filter cannot suppress the ^{15}N -bonded proton, and neither can a single ^1H - ^{15}N REDOR filter for ^{13}C -bonded proton, even when much longer dephasing times were used. Therefore, dREDOR is the most efficient sequence for dephasing all protons, even when mobile functional groups are present, as is common in biological systems. In the rigid-molecule model, when dREDOR filter is applied, the proton magnetization decays completely in 80 μs for CH protons and in 0.14 ms for NH protons. In the mobile-molecule model, the dephasing is much slower, with 0.4 ms dREDOR time needed to dephase the spin magnetization of CH protons and 0.7 ms for NH protons. This is an impressively short time, since the individual ^1H - ^{13}C REDOR and ^1H - ^{15}N REDOR filters require several-fold longer dephasing time than dREDOR to remove the magnetizations of both ^{13}C - and ^{15}N -bonded protons. In addition, the order parameter being one fifth of the rigid limit value for all dipolar interactions results in 5-fold slower decay of dREDOR dephasing curves (400 μs vs. 80 μs , and 0.7 ms vs. 0.14 ms). In the model where only the C-H bond is considered mobile, the ^1H - ^{15}N REDOR decay rate for the CH proton and all REDOR-filtered decay rates for the NH proton are 4–5 fold larger than the rates in the mobile-molecule model, due to the reduction of one-bond ^1H - ^{13}C dipolar coupling by motion. However, the dREDOR and ^1H - ^{13}C REDOR decay rates for CH proton remain unaffected.

Effects of double REDOR filter in proteins: dynein's LC8

To investigate the performance of the dREDOR filter on peptides and proteins, dREDOR-CPMAS, ^1H - ^{13}C REDOR-CPMAS, and ^1H - ^{15}N REDOR-CPMAS spectra were acquired on U- ^{13}C , ^{15}N -LC8 using various REDOR dephasing times. The dephasing data points are fitted to an exponential decay function, same as for histidine, as illustrated in Fig. 4. The dephasing rates of proton magnetizations for the different ^{13}C and ^{15}N spins in LC8 are summarized in Table. 2. Based on the dephasing curves, the dREDOR time required to suppress signals of interest in the ^{13}C spectrum can be readily chosen. As shown in Fig. 4 A-E, the signals in the aliphatic, aromatic and carbonyl regions of the ^{13}C CPMAS spectrum are removed by a dREDOR filter of 0.71 ms ($10\tau_R$, $\nu_R=14$ kHz). The residual signal at 157.2 ppm in the dREDOR-CPMAS spectrum corresponds to the C_γ nucleus of arginine. This sidechain carbon atom does not have a directly bonded proton and requires much

longer recoupling time to render signals completely suppressed. There are three remaining tiny signals in the aliphatic region that might arise from mobile lysine residues. An additional set of dREDOR-based spectra was therefore collected with a presaturation $\pi/2$ -pulse train applied on ^{13}C channel before the proton excitation, to avoid residual signals from the previous scan (named “dREDOR_satC” in Fig. 4 B-E). In the dREDOR- (^{15}N) CPMAS spectrum, all signals that are detected in the conventional ^{15}N CPMAS experiment can be removed with a dREDOR filter of 0.57 ms (Fig. 4G). These results unequivocally demonstrate that the dREDOR is an efficient sequence for the removal of proton magnetization in uniformly ^{13}C and ^{15}N labeled proteins.

Probing intermolecular interfaces with dREDOR based experiments: U- ^{13}C , ^{15}N -CAP-Gly/EB1 complex

We next explored an application of dREDOR-based experiments to probe intermolecular interfaces in protein-protein complexes. We examined U- ^{13}C , ^{15}N -CAP-Gly in complex with natural abundance EB1. As shown in Fig. 5, the magnetization from the protons directly bonded to ^{13}C and ^{15}N sites in CAP-Gly is efficiently dephased by the dREDOR filter, and the intermolecular correlations across the CAP-Gly/EB1 interface can be established by a subsequent polarization transfer from protons in EB1 to ^{13}C or ^{15}N sites in CAP-Gly. As a control, the dREDOR filter was first applied in free U- ^{13}C , ^{15}N -CAP-Gly at different temperatures (Fig. 5A). Since long contact time needs to be used for U- ^{13}C , ^{15}N -CAP-Gly/EB1 complex to establish intermolecular long-distance polarization transfer (see below), the control experiments were also carried out for free CAP-Gly with the CP contact time of 5.0 ms. The analysis of the dephasing behavior is hence based on the dREDOR- (^{13}C) CPMAS spectra of free CAP-Gly acquired with the ^1H - ^{13}C CP contact time of 1.0 ms and varied durations of REDOR dephasing periods (Fig. 5 B-D). As shown in Fig. 5A, the dREDOR dephasing time of 0.71 ms is sufficient to completely remove the ^1H signals that belong to the U- ^{13}C , ^{15}N -CAP-Gly at the sample temperature of -16°C when CP contact time of 5.0 ms is used for the dREDOR- (^{13}C) CPMAS experiment. Nearly all signals are removed in this dREDOR- (^{13}C) CPMAS spectrum except for two signals associated with mobile Lys sidechains, at 43.2 and 29.7 ppm. However, the intensities of these two residual signals are $\sim 15\%$ of those in the dREDOR-CPMAS spectrum of the U- ^{13}C , ^{15}N -CAP-Gly/EB1 complex, indicating that they also constitute the intermolecular contacts (see the below discussion). A few more residual signals were observed in the control experiment acquired at -1°C compared to that at -16°C ; these are likely due to higher mobility of the free CAP-Gly molecule at higher temperature (Yan et al. 2015b). The intensities of these residual signals in dREDOR-CPMAS spectrum of free CAP-Gly are on average $\sim 50\%$ or lower compared to those in the 1D dREDOR-CPMAS spectrum of CAP-Gly/EB1 complex acquired at -1°C , and the corresponding residues also constitute the binding contacts. Expanded views of the dREDOR- (^{13}C) CPMAS spectra of free U- ^{13}C , ^{15}N -CAP-Gly and U- ^{13}C , ^{15}N -CAP-Gly/EB1 as well as their difference spectra are shown in Fig. S3. These residual signals are attenuated in the difference spectra compared to the spectra of complex at both temperatures. We note that different numbers of transients were employed so that the signal-to-noise ratios are similar in the two samples. Similar to LC8, the ^{13}C S/S₀ curves of free CAP-Gly decay nearly completely in 0.7 ms, and at longer times small negative oscillating signals are observed, in line with the expected REDOR dephasing dynamics.

While a dREDOR filter of 0.71 ms removes most of the signals of free CAP-Gly, prominent signals are observed in the 1D dREDOR-(^{13}C)CPMAS spectra (the bottom spectrum in Fig. 5E) of U- ^{13}C , ^{15}N -CAP-Gly/EB1 complex acquired at same experimental conditions as for free CAP-Gly ($T = -16^\circ\text{C}$ and CP contact time of 5.0 ms). This result indicates that the dREDOR filter can selectively dephase ^1H signals from the U- ^{13}C , ^{15}N -enriched protein in the complex whereas the ^1H signals from the unlabeled fragment are retained. To assign the chemical shifts in the 1D spectrum that correspond to direct intermolecular contacts between CAP-Gly and EB1, a 2D dREDOR-CORD spectrum of the complex was recorded (Fig. 6A). On the basis of the cross peak assignments in this 2D spectrum, most of the peaks in the 1D dREDOR-(^{13}C)CPMAS spectrum were unambiguously identified (Fig. S4). Residues that are detected in either 1D dREDOR-CPMAS or 2D dREDOR-CORD are considered to be binding contacts determined by dREDOR. The 1D dREDOR-(^{13}C)CPMAS spectra acquired at -16°C and -1°C reveal a few different signals (e.g. the peaks at ~ 16 ppm, 50–53 ppm and 70–75 ppm, as illustrated in Fig. 5E), which indicate slightly different binding contacts between CAP-Gly and EB1. The 2D dREDOR-CORD experiment in the U- ^{13}C , ^{15}N -CAP-Gly/EB1 complex was carried out using the optimum experimental temperature (-1°C). The dipolar dREDOR transfer dynamics of the interface was recorded as a function of CP contact time, where the dREDOR mixing time was fixed as 0.71 ms (Fig. 5F). The 1D dREDOR-(^{13}C)CPMAS spectra acquired at various CP times are shown in Fig S5. Most signals in the dREDOR-(^{13}C)CPMAS spectra acquired with CP contact times of 0.2 or 0.5 ms have intensities that are discernible from noise and grow monotonously as the contact time is increased. We did not observe new peaks in the spectra acquired with CP contact times longer than 3.0 ms, except for two new resonances, C_α of A45 at 51.2 ppm and $\text{C}_{\delta 1}$ of I87 at 14.8 ppm. This suggests that the ^{13}C signals detected in the dREDOR-(^{13}C)CPMAS spectrum of CAP-Gly/EB1 complex are transferred from natural abundance EB1 and not from U- ^{13}C , ^{15}N -CAP-Gly. If the latter were the case (i.e., the remaining proton magnetizations originated from U- ^{13}C , ^{15}N labeled protein), numerous new signals would have emerged at long CP contact times, given the fact that the CAP-Gly protein is rigid when bound to EB1, which enables efficient long-range magnetization transfer, as we have previously demonstrated (Yan et al. 2015b). In addition, the fits of the dREDOR S/S_0 curves of ^{13}C spins in the complex only decay to a minimum value of ~ 0.45 when the CP contact time is 5.0 ms, indicating the existence of long-distance dipolar couplings and spin-spin correlations.

We next compared the residues of CAP-Gly domain located at the binding interface of CAP-Gly/EB1 as determined by dREDOR-based experiments with those identified on the basis of chemical shift perturbations (CSPs), reported by us previously (Yan et al. 2013a). The results are shown in Fig. 6. The interfaces are color-coded in the primary sequence of CAP-Gly and also mapped on the crystal structure of the heterodimeric CAP-Gly/EB1 protein complex (PDB ID: 2HKQ). The results of the two approaches are generally consistent with several residues that have been reported to be involved in the binding interaction are detected in both cases, such as K68 and G71 in GKNDG motif, V47 and T50 in $\beta 2$ - $\beta 3$ loop and S92 and Q93 in C-terminal α -helix. Yet, many CSPs correspond to residues, which are not revealed by dREDOR; these residues are in spatial proximity with some binding contacts determined by dREDOR (e.g. G30-S31, G39 and V44–45) and experience allosteric effects

but are not involved in the formation of the intermolecular interface. This result is consistent with our prior observations for CAP-Gly/MT complex (Yan et al. 2015a). Thus, dREDOR is a more direct method than CSP for mapping out intermolecular interfaces, especially when the binding affinity is moderate or weak.

The binding interface of CAP-Gly domain with EB1 determined directly by dREDOR comprises mainly the β 3- β 4 loop and S92-Q93 segments of CAP-Gly. Residues V47, T50 and K56 are in close contacts with the EB1 C-terminal α helix while H40-T43 and I61 are in proximity to the β 3- β 4 loop, which contains the GKNDG binding motif. The C-terminal α -helix (S92-Q93) exhibits obvious NMR chemical shifts changes upon complex formation in both the microcrystalline and solution states and was shown to have hydrogen bonding interaction with tripeptide segment EEY at the C-terminal tail of EB1 (Hayashi et al. 2005; Honnappa et al. 2006; Yan et al. 2013a). The rest of the residues detected by the dREDOR-based experiments are located in the β 3- β 4 loop. These results are generally consistent with the previous X-ray diffraction studies (Hayashi et al. 2005; Honnappa et al. 2006). We note that some of these residues detected by dREDOR (i.e. binding contacts in CAP-Gly domain) might be involved in water-mediated interaction with EB1. One example is the Q93 since there are hydrogen-bonding interactions seen between glutamate side chains of EB1 C-terminal tail and Q93/R90 of CAP-Gly domain in previous report (Honnappa et al. 2006). The representative resonances detected by dREDOR experiments and their distances to the binding interface in X-ray structure are summarized in Table S1.

Residues K68 (located in the GKNDG motif) and K66, whose sidechains do not dephase in the reference dREDOR-CPMAS spectrum form intermolecular contacts with EB1 as well, as evidenced by the corresponding peak intensities being 1.8 times higher in the complex than in the free CAP-Gly (with proper signal averaging performed to ensure equivalent signal-to-noise ratios in the two samples). We note that the C-terminal acidic tail of EB1, which is an important binding part for CAP-Gly, is missing in the X-ray structure. The hypothesis for the interaction mode between CAP-Gly and EB1 is that the C-terminal tail passes above the conserved hydrophobic groove that comprises T50, K68, V73 and Q93, and wraps around the β 3- β 4 loop.

DISCUSSION

It is important to note that REDOR filters have been applied by other groups in different contexts as well, since their original introduction by Gullion and Schaefer (Gullion and Schaefer 1989), and a plethora of subsequent studies emerged demonstrating their elegant applications. For instance, the relatively recent MELODI-HETCOR sequence developed by Hong and coworkers that includes a MELODI filter was first proposed for one-bond proton dipolar dephasing and demonstrated on unlabeled amino acids to detect medium- and long-range ^1H - ^{13}C correlations (Yao et al. 2001). The interaction of water with Arg side chains in membrane-bound PG-1 peptide was investigated with two-rotor-period and four-rotor-period MELODI-HETCOR experiments under ^1H - ^{13}C , ^1H - ^{15}N or both ^1H - ^{13}C and ^1H - ^{15}N dipolar dephasing (Li et al. 2010). In this study most experiments were acquired with a dephasing time of 0.29 ms ($2\tau_R$, $\nu_R=6859$ Hz) but to further suppress signals of mobile side chains a 0.58 ms ($4\tau_R$) REDOR dephasing was applied. Subsequently the MELODI-

HETCOR experiment with 640 μ s of ^1H – ^{13}C and ^1H – ^{15}N dephasing and 250 μ s LGCP contact time was employed to probe the water-accessible sites on Pf1 bacteriophage (Sergeyev et al. 2014). Most ^1H signals from the ^{13}C and ^{15}N enriched proteins are fully suppressed with MELODI filter of the chosen length except for some methyl and amino protons. These studies proved the utility of MELODI method and simultaneous ^{13}C and ^{15}N dephasing for detection of water-interactions of membrane proteins.

In contrast to MELODI-HETCOR, the dREDOR sequence applied in our work utilizes the dREDOR filter of adjustable time by varying the number of REDOR dephasing periods without homonuclear decoupling during the dREDOR filter. These differences may affect the dephasing dynamics profile of ^1H magnetization and the sensitivity of dREDOR filter might be different from that probed with the MELODI filter. In this work the dREDOR-filter with simultaneous ^{13}C and ^{15}N dephasing is optimized to probe protein-protein interaction. In addition, the conventional ramp-CP is used instead of the LG-CP for the heteronuclear H-X polarization transfer following the double REDOR filter in our experiments. Although the LGCP is efficient for suppressing homonuclear couplings and selective polarization transfer, the conventional double REDOR filter and RAMP-CP discussed here are necessary because they ensure quasi-uniform dipolar dephasing during the filter time and provide significant gains in sensitivity, which is essential for biological systems of large size such as those investigated in this work.

We note that it is possible that water-protein interactions may in principle contribute to the dREDOR-based spectra of biological complexes. However, the presence of such water-protein contacts can be easily probed by the dREDOR-HETCOR experiments and indicated by signals that appear at ^1H chemical shift of water in the dREDOR-HETCOR spectra. The possibility of this kind of interaction was examined by us previously (Yan et al. 2015a). In that study, the 2D dREDOR-HETCOR experiment was conducted in the $\text{U-}^2\text{H}$, ^{13}C , ^{15}N -CAP-Gly/microtubule complex prepared in 99.8% D_2O , and there was no evidence for water-protein interaction. Therefore, there are no possible artifacts due to bound water for CAP-Gly/EB1 complex after the application of double REDOR filter.

Taken together, our results establish the usefulness of simultaneous ^1H – ^{13}C and ^1H – ^{15}N REDOR dephasing filters in interpreting protein-protein interactions. The systematic investigation of dREDOR dephasing dynamics lays the basis for incorporating the dREDOR filters into various 1D and 2D experiments, such as dREDOR-CPMAS and dREDOR-CORD. The binding interface of EB1 on CAP-Gly derived by dREDOR based experiments is comprised of several key EB1-binding segments of CAP-Gly, which is generally consistent with the findings from the chemical shift perturbation analysis; albeit the latter experiments also report on allosteric structural changes upon binding that may occur at positions distal from the binding interfaces. The double REDOR method analyzed here is broadly applicable to investigations of intermolecular interfaces formed by binding partners in a wide range of systems and can be combined with many other NMR experiments for establishing different types of correlations following the dREDOR filter.

CONCLUSIONS

The dREDOR-based experiments with simultaneous ^1H – ^{13}C or ^1H – ^{15}N dipolar recoupling are utilized for structural studies of protein-protein binding interfaces in complexes comprised of a $\text{U-}^{13}\text{C},^{15}\text{N}$ -enriched protein and its natural abundance binding partner. The dephasing and polarization transfer dynamics of dREDOR filter were analyzed with Histidine and LC8 protein, demonstrating the effectiveness of this method in suppressing directly ^{13}C - and ^{15}N -bonded protons. The utility of 1D dREDOR-CPMAS and 2D dREDOR-CORD experiments for probing intermolecular interaction sites was demonstrated on $\text{U-}^{13}\text{C},^{15}\text{N}$ -CAP-Gly/EB1. The binding interface of EB1 on CAP-Gly derived by dREDOR based experiments is consistent with the X-ray crystal structure. The dREDOR method is found to be a more direct approach for intermolecular interfaces compared to chemical shift perturbation analysis since the latter also reflects allosteric structural changes that may occur far from the binding interfaces. More broadly, the double REDOR based approach can be applied to any protein complexes beyond the examples presented in this study and is anticipated to be particularly beneficial for large biological assemblies in the context of cells or cell fragments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Pulse sequences for the double-REDOR experiments incorporating simultaneous ^1H - ^{13}C and ^1H - ^{15}N dipolar recoupling: **A)** 1D dREDOR with direct ^1H detection; **B)** 1D dREDOR- (^{13}C)CPMAS and dREDOR- (^{15}N)CPMAS; **C)** 2D dREDOR-CORD for ^{13}C - ^{13}C correlations. XY-8 phasing scheme was applied in the rotor-synchronized REDOR π -pulse train. A pre-saturation $\pi/2$ -pulse train is applied on the ^{13}C channel before the proton excitation to remove remaining signals from the previous scan. The 1D dREDOR-based experiments recorded in this work are without pre-saturation except where specified. The complete phase cycle is given in the Supporting Information.

Figure 2.

1D MAS spectra and dREDOR dephasing dynamics in $U\text{-}^{13}\text{C}, ^{15}\text{N}$ -Histidine. **A-D)** Experimental ^1H - ^{13}C , ^1H - ^{15}N and double REDOR dephasing curves for the ^{13}C atoms (**A-C**) and 1D $(^{13}\text{C})\text{CPMAS}/\text{dREDOR}$ - $(^{13}\text{C})\text{CPMAS}$ spectra (**D**). **E)** Ball-and-stick representations of the histidine molecule in the biprotonated state (+1, left) and neutral τ tautomer state (τ , right). The ratio of two protonation states +1: τ in the sample under investigation is approximately 3:1. **F-H)** Experimental REDOR dephasing curves of ^{15}N atoms (**F-G**) and 1D $(^{15}\text{N})\text{CPMAS}/\text{dREDOR}$ - $(^{15}\text{N})\text{CPMAS}$ spectra (**H**). **I-J)** REDOR

dephasing curves of ^1H atoms (**I**) and 1D ^1H /dREDOR- ^1H spectra (**J**). The experimental dephasing data points are fitted to exponential decay function (see notes in Table 1). The dephasing curves for all resonances that have resolved peaks are shown in Fig. S1. All spectra were acquired at 19.96 T with the MAS spinning frequency of 20 kHz. The contact time for both (^{13}C)CPMAS and (^{15}N)CPMAS was 1 ms.

Figure 3.

Simulated ^1H - ^{13}C (blue), ^1H - ^{15}N (red) and double REDOR (black) dephasing curves for protons with directly bonded ^{13}C or ^{15}N atoms in an isolated ^1H - ^{13}C - ^{15}N - ^1H spin system. Rigid molecule (A and B) partially mobile (C and D) and mobile (E and F) models were used. In the partially mobile model the dipolar order parameter for ^1H - ^{13}C bond was set to 0.2 while other bonds were assumed to be rigid with order parameter of 1.0. The ^1H spin marked in red dashed circle is observed. The simulations for the mobile spin model were carried out with both ^1H - ^{13}C and ^1H - ^{15}N dipolar order parameters at 0.2. It should be noted that the dipolar dephasing by dREDOR will not work if the motions are infinitely fast on the timescale of dipolar couplings ($S^2 \sim 0$).

Figure 4.

1D MAS spectra and dREDOR dephasing dynamics in U- ^{13}C , ^{15}N -LC8 doped with 5 mM Cu-EDTA. **A-E**) 1D (^{13}C)CPMAS/dREDOR-(^{13}C)CPMAS spectra of (A) and REDOR dephasing curves of ^{13}C atoms based on the integrals of different chemical shift regions (B-E). **F-G**) 1D (^{15}N)CPMAS/dREDOR-(^{15}N)CPMAS spectra (F) and REDOR dephasing curves of backbone ^{15}N atoms (G). Bulk backbone ^{15}N signals are fully suppressed with 0.57 ms dREDOR dephasing. The dephasing data points are fitted to exponential decay function. All spectra were acquired at 19.96 T with the MAS spinning frequency of 14 kHz. The contact time for both (^{13}C)CPMAS and (^{15}N)CPMAS was 1 ms.

Figure 5.

1D MAS spectra and dREDOR dephasing dynamics in $\text{U-}^{13}\text{C}$, ^{15}N -CAP-Gly and $\text{U-}^{13}\text{C}$, ^{15}N -CAP-Gly/n.a. EB1 complex. **A-D**) 1D ^{13}C CPMAS and dREDOR- ^{13}C CPMAS spectra (A) and REDOR dephasing curves for different types of ^{13}C atoms (**B-D**) in free $\text{U-}^{13}\text{C}$, ^{15}N -CAP-Gly. The analysis of the dephasing behavior is based on the dREDOR- ^{13}C CPMAS spectra acquired with the $^1\text{H-}^{13}\text{C}$ CP contact time of 1.0 ms and varied durations of REDOR dephasing periods. The majority of the signals are removed with dREDOR dephasing time of 0.71 ms at -16°C , except for two signals from mobile lysine residues. **E-G**) 1D

(^{13}C)CPMAS and dREDOR-(^{13}C)CPMAS spectra of U- ^{13}C , ^{15}N -CAP-Gly/n.a. EB1 complex (**E**); the REDOR dipolar transfer dynamics of the interface was recorded as a function of CP contact time, where the dREDOR mixing time was fixed as 0.71 ms (**F**); experimental dREDOR dephasing curves of ^{13}C atoms of CAP-Gly (**G**) in U- ^{13}C , ^{15}N -CAP-Gly/n.a. EB1 complex. In contrast to free CAP-Gly, in the CAP-Gly/EB1 complex, several relatively intense signals were retained and detected in dREDOR spectra with 0.71 ms dephasing time. Expanded views of the dREDOR-(^{13}C)CPMAS spectra of free U- ^{13}C , ^{15}N -CAP-Gly and U- ^{13}C , ^{15}N -CAP-Gly/EB1 as well as their difference spectra are shown in Fig. S3. The spectra were acquired at magnetic field of 11.7 T with the MAS frequency of 14 kHz.

A. Top: 2D dREDOR-CORD (grey) and regular DARR (green) spectra of U- ^{13}C , ^{15}N -CAP-Gly/n.a. EB1. The ^{13}C - ^{13}C mixing time is 50 ms in both experiments. Assignments of dREDOR-based CORD spectrum are labeled in grey. **Bottom:** The primary sequence of CAP-Gly with residues color-coded as follows: residues detected in dREDOR experiment of CAP-Gly/EB1, cyan; residues with chemical shift perturbations (> 0.5 ppm) in CAP-Gly/EB1 compared to free CAP-Gly, yellow. **B.** EB1-binding contacts in CAP-Gly mapped onto the X-ray structure of dimeric CAP-Gly/EB1 (2HKQ). Intermolecular interface determined by chemical shift perturbations of CAP-Gly upon binding to EB1 is shown in yellow (left) and the interface derived by dREDOR-based experiments is shown in cyan (right). The 2D DARR spectrum of U- ^{13}C , ^{15}N -CAP-Gly/EB1 complex was acquired at

19.96 T with sample temperature at +4 °C and the 2D dREDOR-CORD spectrum was acquired at 11.7 T with sample temperature at −1 °C.

Table 1.

Dephasing rate constants of ^1H magnetization in $\text{U-}^{13}\text{C}$, ^{15}N -Histidine by H-N REDOR, H-C REDOR and dREDOR

Atom	Inverse time constant $1/\tau$ (s^{-1}) ^a (coefficient value $\pm$ standard deviation)		
	H-N REDOR	H-C REDOR	dREDOR
C' (+1) ^b	2.8 $\pm$ 0.2	4.1 $\pm$ 0.2	6.1 $\pm$ 0.4
C' (τ) ^b	2.2 $\pm$ 0.4	5.2 $\pm$ 0.4	6.5 $\pm$ 0.4
Ca (+1)	1.5 $\pm$ 0.2	6.9 $\pm$ 0.8	9.7 $\pm$ 1.4
Ca (τ)	1.1 $\pm$ 0.3	9.2 $\pm$ 1.0	11.2 $\pm$ 0.8
C β (+1)	1.6 $\pm$ 0.2	6.6 $\pm$ 0.7	9.5 $\pm$ 1.3
C β (τ)	0.3 $\pm$ 0.3	8.0 $\pm$ 1.7	8.5 $\pm$ 1.0
C γ (+1)	2.8 $\pm$ 0.1	5.1 $\pm$ 0.2	7.6 $\pm$ 0.5
C γ (τ)	1.3 $\pm$ 0.4	5.2 $\pm$ 0.2	8.5 $\pm$ 0.5
C δ 2 (+1)	0.9 $\pm$ 0.1	11.3 $\pm$ 0.9	14.6 $\pm$ 1.6
C δ 2 (0, τ)	0.4 $\pm$ 0.2	14.4 $\pm$ 3.2	15.8 $\pm$ 3.3
C ϵ 1 (+1)	1.5 $\pm$ 0.2	10.1 $\pm$ 0.4	13.5 $\pm$ 1.1
C ϵ 1 (0, τ)	1.3 $\pm$ 0.4	13.0 $\pm$ 2.5	16.0 $\pm$ 2.6
N δ 1 (0, τ)	1.2 $\pm$ 0.0	8.9 $\pm$ 9.1	14.3 $\pm$ 0.4
N δ 1 (+1)	7.1 $\pm$ 1.4	4.1 $\pm$ 0.4	11.1 $\pm$ 0.5
Ne2 (+1)	10.1 $\pm$ 1.9	3.5 $\pm$ 0.5	14.1 $\pm$ 0.9
Ne2 (τ)	8.5 $\pm$ 1.6	4.5 $\pm$ 0.4	15.1 $\pm$ 1.7
Na (+1)	2.7 $\pm$ 0.7	2.3 $\pm$ 0.5	5.8 $\pm$ 0.6
Na (τ)	1.1 $\pm$ 1.2	2.2 $\pm$ 0.3	5.2 $\pm$ 2.0
H δ 1 (+1)	8.9 $\pm$ 1.2	4.1 $\pm$ 0.5	9.1 $\pm$ 0.5
He2 (+1)	10.7 $\pm$ 1.5	3.9 $\pm$ 0.8	11.6 $\pm$ 0.8
Hmulti	1.6 $\pm$ 0.5	2.6 $\pm$ 0.8	3.5 $\pm$ 0.5

^aDephasing curves are fitted to exponential decay function: $y = y_0 + A \exp(-x/\tau)$ τ is the time constant.

^b+1 is the protonated state and τ is the neutral tautomer state. (Li and Hong 2011)

Table 2.

Dephasing rate constants of ^1H magnetization for different types of ^{13}C and ^{15}N spins in $\text{U-}^{13}\text{C}, ^{15}\text{N-LC8}$, $\text{U-}^{13}\text{C}, ^{15}\text{N-CAP-Gly}$ and $\text{U-}^{13}\text{C}, ^{15}\text{N-CAP-Gly/n.a. EB1}$ complex

$\text{U-}^{13}\text{C}, ^{15}\text{N-LC8}$				
Peak regions	Inverse time constant $1/\tau$ (coefficient values $\pm$ standard deviation) (s^{-1}) ^a			
	N-H REDOR	C-H REDOR	dREDOR	dREDORsatC
$^{13}\text{C}^{\alpha'}$	9.6 ± 1.9	5.3 ± 2.2	10.8 ± 5.0	9.4 ± 1.7
$^{13}\text{C}\alpha$	0.2 ± 0.6	9.8 ± 2.2	11.3 ± 3.5	6.9 ± 1.1
Aromatic ^{13}C		17.5 ± 1.4	25.3 ± 6.2	
Sidechains ^{13}C	0.2 ± 0.1	8.3 ± 1.6	8.9 ± 2.0	6.2 ± 0.6
Backbone ^{15}N	17.8 ± 6.6		22.1 ± 4.3	
$\text{U-}^{13}\text{C}, ^{15}\text{N-CAP-Gly}$				
Peak regions	Inverse time constant $1/\tau$ (coefficient values $\pm$ standard deviation) (s^{-1})			y_0
	N-H REDOR	C-H REDOR	dREDOR	dREDOR
$^{13}\text{C}^{\alpha'}$		6.8 ± 0.3	11.3 ± 1.9	
$^{13}\text{C}\alpha$		7.8 ± 1.2	7.5 ± 2.2	-0.1 ± 0.1
Sidechains ^{13}C		8.2 ± 0.8	8.7 ± 1.4	
$\text{U-}^{13}\text{C}, ^{15}\text{N-CAP-Gly/EB1}$				
Peak regions	Inverse time constant $1/\tau$ (coefficient values $\pm$ standard deviation) (s^{-1})			y_0
	N-H REDOR	C-H REDOR	dREDOR	dREDOR
$^{13}\text{C}\alpha$			5.9 ± 2.0	0.4 ± 0.0

^aDephasing curves are fitted to exponential decay function: $y = y_0 + A \exp(-x/\tau)$ τ is the time constant.