



Universiteit
Leiden
The Netherlands

The relation between dynamics and activity of phospholipase A/acyltransferase homologs

Chatterjee, S.D.

Citation

Chatterjee, S. D. (2022, March 2). *The relation between dynamics and activity of phospholipase A/acyltransferase homologs*. Retrieved from <https://hdl.handle.net/1887/3277998>

Version: Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/3277998>

Note: To cite this publication please use the final published version (if applicable).

5

Chapter

Removal of slow-pulsing artifacts in in-phase ^{15}N relaxation dispersion experiments using broadband ^1H decoupling

The work in this chapter was published as: Chatterjee, S. D., Ubbink, M., and van Ingen, H. (2018) Removal of slow-pulsing artifacts in in-phase ^{15}N relaxation dispersion experiments using broadband ^1H decoupling. *J. Biomol. NMR* 71, 69–77.

Abstract

Understanding of the molecular mechanisms of protein function requires detailed insight into the conformational landscape accessible to the protein. Conformational changes can be crucial for biological processes, such as ligand binding, protein folding, and catalysis. NMR spectroscopy is exquisitely sensitive to such dynamic changes in protein conformations. In particular, Carr–Purcell–Meiboom–Gill (CPMG) relaxation dispersion experiments are a powerful tool to investigate protein dynamics on a millisecond time scale. CPMG experiments that probe the chemical shift modulation of ^{15}N in-phase magnetization are particularly attractive, due to their high sensitivity. These experiments require high power ^1H decoupling during the CPMG period to keep the ^{15}N magnetization in-phase. Recently, an improved version of the in-phase ^{15}N -CPMG experiment was introduced, offering greater ease of use by employing a single ^1H decoupling power for all CPMG pulsing rates. In these experiments however, incomplete decoupling of off-resonance amide ^1H spins introduces an artefactual dispersion of relaxation rates, the so-called slow-pulsing artifact. Here, we analyze the slow-pulsing artifact in detail and demonstrate that it can be suppressed through the use of composite pulse decoupling (CPD). We report the performances of various CPD schemes and show that CPD decoupling based on the $90_x\text{--}240_y\text{--}90_x$ element results in high-quality dispersion curves free of artifacts, even for amides with high ^1H offset.

Introduction

Biological macromolecules such as nucleic acids and proteins are non-rigid entities that can populate a variety of conformers in their energy landscape^{1–3}. The lowest energy conformation,

the ground state, is often able to transiently access higher-energy conformations. Even when their population is low ($< 10\%$) and life times is short ($\sim \text{ms}$), these excited states can be crucial for biologically important processes such as enzyme catalysis^{4–8}, ligand binding or protein-protein interaction^{9–14}, and protein folding^{15–20}. While these states cannot be detected directly due to their transient and lowly populated nature, NMR experiments^{21–23} are uniquely able to provide a detailed, atomistic description of the energy landscape. In particular, relaxation dispersion and chemical exchange saturation transfer experiments are particularly powerful herein, as they give access to the population, life times and structures of excited states^{24–31}.

In Carr–Purcell–Meiboom–Gill (CPMG) relaxation dispersion experiments, the characterization of the minor state is derived from the major state peaks by measurement of their effective transverse relaxation rate $R_{2,\text{eff}}$, as a function of the pulsing rate in the CPMG period. Signals of nuclear spins that experience exchange between states with different chemical shifts are affected by exchange-induced line broadening, an effect that depends on the free precession interval ($2\tau_{\text{cp}}$) between the refocusing pulses in the CPMG element^{24,27}. Analysis of the resulting relaxation dispersion curve, a plot of the $R_{2,\text{eff}}$ versus CPMG frequency ($1/4\tau_{\text{cp}}$), allows determination of the rate of exchange (k_{ex}), population of minor state (p_{b}) and the absolute chemical shift difference ($|\Delta\omega|$) between the exchanging states. Importantly, since the shape of the dispersion profile depends on $\Delta\omega$, data is typically acquired at two fields to accurately determine the exchange parameters²⁷.

The ^{15}N backbone amide spin is the most popular nucleus for CPMG RD experiments, due to the simplicity of isotope-labeling, the straightforwardness of the two-spin ^1H – ^{15}N spin system, and the high sensitivity and resolution afforded by these experiments. A critical aspect of these experiments is appropriate handling of differences in the intrinsic R_2 of the in-phase ($N_{x,y}$) and anti-phase ($2N_{x,y}H_z$) ^{15}N magnetization which are generated in the free evolution periods. Anti-phase terms have higher intrinsic relaxation rates due to a contribution of ^1H spin flips to their decay. The original implementation of the ^{15}N CPMG RD experiment uses a relaxation-compensation scheme to average the $N_{x,y}$ and $2N_{x,y}H_z$ relaxation rates³². The ^{15}N CPMG sequence of Hansen *et al.*³³ (CW–CPMG) measures the dispersion profile of pure in-phase $N_{x,y}$ by applying high-power

continuous wave (CW) ^1H decoupling during the CPMG train, offering enhanced sensitivity for non-deuterated proteins³³. Recently, Jiang *et al.*³⁴ modified this sequence (ST–CW–CPMG) to use a single CPMG train with the Yip and Zuiderweg phase cycle (2004) and a single CW decoupling power, yielding dispersion curves free of off-resonance artifacts for a wider range of ^{15}N offset frequencies³⁵.

Both CW–CPMG sequences are nevertheless sensitive to artifacts from ^1H off-resonance effects^{33,35}. Amide ^1H spins that are far off-resonance from the CW decoupling field are not fully decoupled from the ^{15}N spin, resulting in generation of $2N_{xy}H_z$ magnetization through the residual J -coupling. Consequently, higher $R_{2,\text{eff}}$ values will be measured for low ν_{CPMG} values, for which free precession periods are long and more of the antiphase terms will be generated. This so-called slow-pulsing artifact shows up as an artefactual dispersion curve, interfering with accurate extraction of minor-state parameters.

Here, we analyze the slow-pulsing artifact in ^{15}N CW–CPMG sequences in detail and demonstrate a simple method for its removal. In that, we took inspiration from the work of Chakrabarti *et al.*³⁶, where composite pulse decoupling (CPD) was used to suppress ^1H off-resonance effects in exchange mediated saturation transfer experiments. We investigated the performance of various CPD schemes in CW–CPMG sequences and demonstrate here that high power CPD based on the $90_x\text{--}240_y\text{--}90_x$ element achieves artifact-free dispersion curves over a wide range of ^1H offsets.

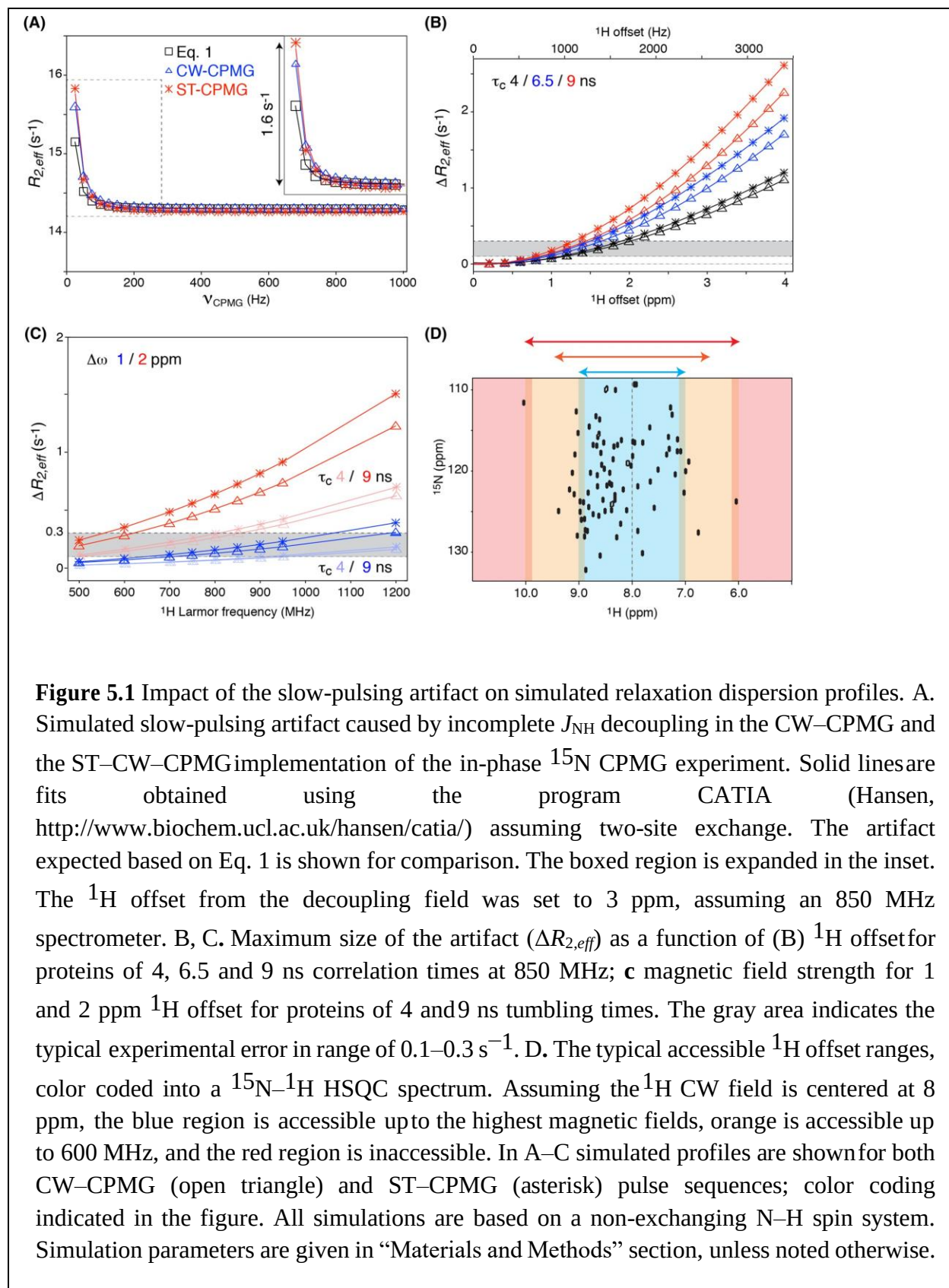
Results

Measurement of in-phase ^{15}N CPMG relaxation dispersion profiles critically relies on H decoupling to measure the pure in-phase $N_{x,y}$ relaxation rate without contamination by the anti-phase relaxation rate. As pointed out in the work of Jiang *et al.*³⁴, the decoupling field strength has a practical limit of roughly 14 kHz, resulting in a residual J coupling interaction for amide protons at non-zero offset to the decoupling field. This interaction causes slow interconversion of in-phase and anti-phase magnetization during the CPMG period, which will lead to undesired averaging of the in-phase and anti-phase relaxation rates^{32,33}. To first approximation, this averaging can be described by the equation derived by Palmer *et al.*⁴² for calculating the effective relaxation rate in spin echo sequences. Here, it is adapted and reformulated to express to the size of the slow-pulsing artifact A :

$$A = \frac{1}{2}(R_2^{anti} - R_2^{in})(1 - \text{sinc}\pi J_r 2\tau_{cp}) \quad (1)$$

where R_2^{in} and R_2^{anti} are the ^{15}N in-phase and anti-phase transverse relaxation rates, J_r is the residual J -coupling, and $2\tau_{cp}$ is the inter-pulse delay in the CPMG pulse train. In the limit of perfect decoupling $J_r \approx 0$, the sinc factor approaches 1 and $A \approx 0$ for all τ_{cp} values. For non-zero J_r , A approaches zero in the limit of fast pulsing where τ_{cp} is very small. For slow pulsing, however, there is a non-zero artifact, with a theoretical limit of $0.5 (R_{2,anti} - R_{2,in})$ for infinitely slow pulsing. In practice, J_r can be as much 16 Hz (for 3 ppm ^1H offset at 850 MHz) and $2\tau_{cp}$ is typically at most 20 ms, which would generate a maximum artifact of roughly 10% of the difference between the anti-phase and in-phase relaxation rate.

To assess more precisely how the slow pulsing artifact is manifested in ^{15}N CPMG–CW and ST–CW experiments, numerical simulations of these sequences were performed in Liouville space for a non-exchanging two spin N–H system. Figure 5.1A compares the obtained dispersion profiles for the two experiments with the predicted curve based on Eq. 1, for a N–H system with 3 ppm ^1H offset at an 850 MHz spectrometer. Whereas a flat curve is expected for a non-exchanging system, systematically increased $R_{2,eff}$ values are measured in the slow pulsing regime for both pulse sequences. While Eq. 1 is derived for periods of free evolution in absence of a decoupling field, the curvature of the slow-pulsing artifact matches the predicted sinc dependence on the pulsing rate.



The size of the artifact is somewhat underestimated by Eq. 1. The original CW sequence shows slightly lower sensitivity to the artifact than the ST–CW experiment. This difference can be traced back to presence of the ^{15}N refocusing pulse in between the two halves of the total CPMG period in the CW experiment. Importantly, since the shape of the artifact is virtually indistinguishable from a *bona-fide* dispersion profile, the artefactual $R_{2,\text{eff}}$ values can be fitted to an actual dispersion curve (see solid lines in Figure 5.1A), illustrating the potential impact on the extracted exchange parameters.

Since the size of the slow pulsing artifact is governed by the relaxation difference between in-phase and anti-phase magnetization, it is dependent on protein size. Large proteins have more efficient ^1H – ^1H spin flips which increase the anti-phase relaxation rate. Figure 5.1B compares the magnitude of the artifact for three different protein sizes as function of ^1H offset from the decoupling field. For larger proteins, where the chance of finding amide protons at high offset is also higher, the artifact can be well above 1 s^{-1} . At offsets larger than $\sim 1000\text{ Hz}$ the slow pulsing artifact will be higher than the typical experimental error (on the order of $0.1\text{--}0.3\text{ s}^{-1}$)⁴³, as also noted by Jiang *et al.*³⁴.

Since relaxation dispersion data need to be acquired at two magnetic fields in order to extract accurate protein dynamics parameters, we compared the size of the slow pulsing artifact for amide groups at 1 and 2 ppm ^1H offset as function of magnetic field strength in Figure 5.1C. High field strengths are not only attractive because of the sensitivity and resolution they afford, but also because they are more sensitive to exchange processes as they increase the frequency difference between states, $\Delta\omega$. However, for a given resonance, the offset from the decoupling field, and thus the slow pulsing artifact, will increase with increasing magnetic field strength. Strikingly, the artifact will already be significant at 1 ppm offsets for medium-sized proteins in a future 1.2 GHz spectrometer. To illustrate the impact of the slow pulsing artifact, generated by the inability of CW irradiation to decouple the full width of the amide spectrum, the HSQC can be divided in three areas: a narrow region $\pm \sim 1\text{ ppm}$ around the carrier frequency of the decoupling field that will be free of significant artifacts, the region beyond $\pm \sim 2\text{ ppm}$ in which significant artifacts will

already occur at the lowest typical field strength, and the intermediate region (Figure 5.1D). To confirm the results obtained from simulations, we experimentally demonstrated the problem using the ST–CW–CPMG pulse sequence on a sample containing azurin, a 16 kDa electron transfer metalloprotein⁴⁴. A small subset of residues in azurin have been reported to undergo conformational exchange on the millisecond timescale⁴⁵. To emphasize the slow pulsing artifact, we purposely centered the ^1H decoupling field at 16 ppm such that the dispersion profiles are dominated by the artifact (Figure 5.2A). Using this setup, we next screened several broadband decoupling sequences for their ability to suppress the artifact. These sequences rely on composite pulses to offer good population inversion even in the presence of off-resonance effects⁴⁶, and thus should be able to suppress the artifact in theory. As can be seen in Figure 5.2B, a wide range of CPD schemes indeed suppressed the artifact. Notably, the use of GARP⁴⁷ and DIPSI2⁴⁸ results in spurious elevated $R_{2,\text{eff}}$ values at high pulsing rates, rendering the dispersion curves unusable. These spikes originate from the timing mismatch between the continuous train of (composite) 180° pulses on the ^1H channel on the one hand and the repetition of free-evolution and 180° refocusing pulses on the ^{15}N channel on the other hand. This mismatch results in incomplete decoupling at the end of each τ_{cp} period and thus elevated $R_{2,\text{eff}}$ values (Figure 5.2C). As noted by Jiang *et al.*³⁴, the duration of the mismatch is short when using adequately high power CW ^1H decoupling, and thus the effect is small. Both DIPSI2 and GARP use particularly long composite pulses (corresponding to the length of 2590° and 1054° rotation, respectively), which aggravates the impact of the timing mismatch, in particular at high pulsing rates, where the effects from each τ_{cp} period are compounded. Indeed, use of WALTZ (540° duration)⁴⁹ and MLEV (360° duration)^{50–52} with shorter duration of the composite pulse did not cause such high spikes. We next applied the 90_x – 240_y – 90_x CPD scheme, which was recently used to suppress artifacts from incomplete ^1H decoupling in exchange mediated saturation transfer experiments³⁶. The 90_x – 240_y – 90_x CPD sequence has a short overall duration (420° rotation) and offers relatively broadband inversion, free from off-resonance effects without relying on supercycles^{52,53}. Gratifyingly, the 90_x – 240_y – 90_x sequence effectively eliminated the artifacts without causing appreciable spikes or scatter in $R_{2,\text{eff}}$ values (Figure 5.2D). The requirement for a short duration of the CPD element also means that the broadband performance of CPD decoupling cannot be used to reduce the decoupling power. Tests showed that reducing the decoupling power to 7 kHz (34 μs decoupling pulse) resulted

in spurious artifacts dominating the dispersion curves at high CPMG pulsing rates (data not shown).

While successful in suppressing the slow-pulsing artifacts, the use of composite pulse sequences for decoupling results in systematic differences in $R_{2,eff}$ values compared to those obtained using CW decoupling. This is most apparent from the WALTZ data in Figure 5.2B, showing systematically reduced $R_{2,eff}$ values compared to the CW reference data. Such offsets between the CPD-derived and CW-derived dispersion curve are also found for MLEV and $90_x-240_y-90_x$ decoupling, although typically much smaller. When using the $90_x-240_y-90_x$ sequence, the average offset over all residues was found to be $\sim 0.3 \text{ s}^{-1}$ with 90% of the profiles having offsets below 0.6 s^{-1} (see Supplemental Table S5.1). Since this offset is small and the absolute value of $R_{2,eff}$ is not of importance when fitting dispersion curves, it will have negligible impact on the usefulness of the data obtained with CPD decoupling schemes.

Having established that WALTZ, MLEV and $90_x-240_y-90_x$ decoupling sequences are able to suppress the slow pulsing artifact, we further tested their efficacy in a regular experimental setup with the decoupling field centered at 8.2 ppm. The obtained $R_{2,eff}$ values were compared point-by-point between the CPD and the CW data-set, and the root-mean-square deviation (RMSD) between data sets was calculated with and without compensating for the systematic offset in $R_{2,eff}$ values between the two datasets (Figure 5.3A). Clearly, the $90_x-240_y-90_x$ sequence performs best with an average RMSD to the reference CW data set of 0.17 s^{-1} , which is on the order of the experimental error. The high quality of the data is visible from comparison of profiles obtained for residues with negligible ^1H offset, such as shown in Figure 5.3B. At high ^1H offset from the decoupling field, the CW data suffers from the slow pulsing artifact, which is absent when using the $90_x-240_y-90_x$ CPD sequence, as exemplified for T52 in Figure 5.3C. Notably, this residue shows the slow pulsing artifact superimposed on a genuine dispersion of $R_{2,eff}$ values. From the comparison to the CPD-based experiment, it becomes clear that the data point at 25 Hz ν_{CPMG} pulsing rate is strongly affected by the slow pulsing artifact with $R_{2,eff}$ value spuriously elevated by $\sim 1 \text{ s}^{-1}$. As a final experiment, we recorded both CW and CPD-based dispersion profiles at the national ultra-high field NMR Facility at 950 MHz. At this field, the resonance with the

highest ^1H offset shows a slow-pulsing artifact of $\sim 1.5 \text{ s}^{-1}$ in the CW experiment, which is effectively suppressed when using the $90_x-240_y-90_x$ decoupling sequence (Figure 5.3D).

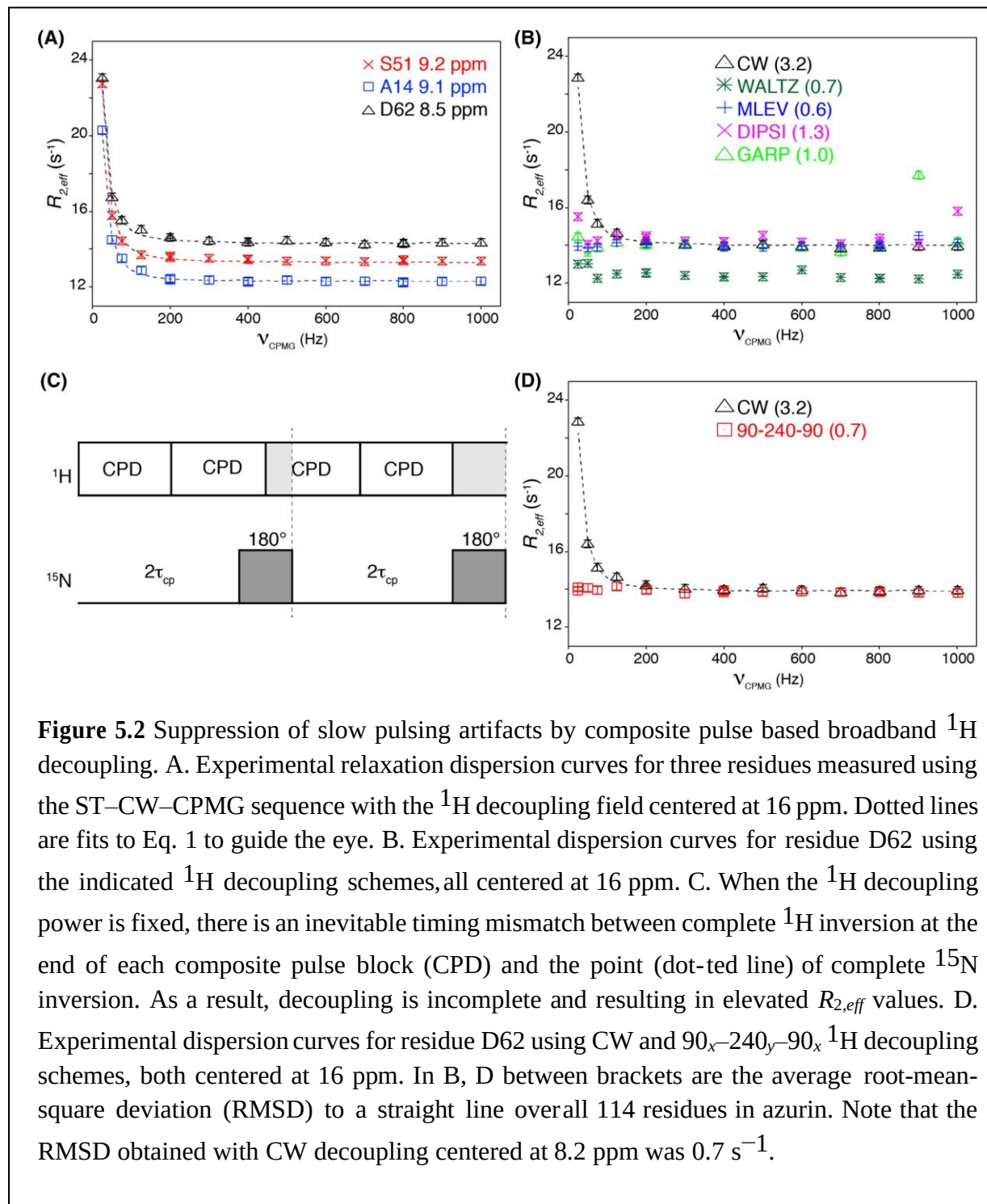


Figure 5.2 Suppression of slow pulsing artifacts by composite pulse based broadband ^1H decoupling. A. Experimental relaxation dispersion curves for three residues measured using the ST–CW–CPMG sequence with the ^1H decoupling field centered at 16 ppm. Dotted lines are fits to Eq. 1 to guide the eye. B. Experimental dispersion curves for residue D62 using the indicated ^1H decoupling schemes, all centered at 16 ppm. C. When the ^1H decoupling power is fixed, there is an inevitable timing mismatch between complete ^1H inversion at the end of each composite pulse block (CPD) and the point (dot-ted line) of complete ^{15}N inversion. As a result, decoupling is incomplete and resulting in elevated $R_{2,eff}$ values. D. Experimental dispersion curves for residue D62 using CW and $90_x-240_y-90_x$ ^1H decoupling schemes, both centered at 16 ppm. In B, D between brackets are the average root-mean-square deviation (RMSD) to a straight line overall 114 residues in azurin. Note that the RMSD obtained with CW decoupling centered at 8.2 ppm was 0.7 s^{-1} .

Discussion

We have investigated the impact of the slow-pulsing artifact in in-phase ^{15}N relaxation dispersion experiments by theoretical considerations, numerical simulations and experiments. We show that the artifact can be removed by using CPD-based ^1H decoupling during the CPMG period. Out of the tested CPD sequences, the $90_x-240_y-90_x$ sequence offers the best performance: the artifact is fully suppressed, while retaining shape of the dispersion curve obtained using CW decoupling within experimental error. Notably, this is done without introducing spurious spikes in $R_{2,\text{eff}}$ values at high pulsing rates, and with minimal offset to the CW-based dispersion profiles. Critical to its performance seems to be short duration of the composite pulse combined with relatively high quality of off-resonance performance.

The cause of the slight offset between the CPD and CW- based dispersion profiles is unclear. Closer inspection shows that the magnitude of the offset shows no correlation to either the N , H_N , or H_α chemical shift and that both reference (no CPMG delay) and dispersion experiment (with CPMG delay) have slightly altered intensities ($\sim 2-5\%$) in the CPD experiment compared to the CW experiment. The effect on the reference experiment, where the decoupling block is carried out before the recycle delay, signifies that the both types of decoupling result in a different steady-state magnetization, presumably both for water and protein protons.

As for the water magnetization, a disadvantage of using CPD over CW decoupling is the loss of control over its state. Whereas in the CW case the water magnetization is spin-locked and returned to $+z$ after the CPMG period, continuous alteration between x and y -pulse phase during the $90_x-240_y-90_x$ CPD element causes dephasing and loss of water polarization. Experimental tests Hiller *et al.*⁵⁴ demonstrate this effect and show that after a 2 s delay, corresponding to the recycle delay to the next proton excitation pulse, there is minimal difference between the water polarization in the CPD and CW case (see Supplemental Figure S5.1). Here, radiation damping caused by the high Q of the cryogenic probe likely aids the recovery of the water magnetization in the CPD case. Additionally, the low pH of the sample (5.5) will slow down amide-water exchange and thus additionally dampen the effect of (residual) water saturation.

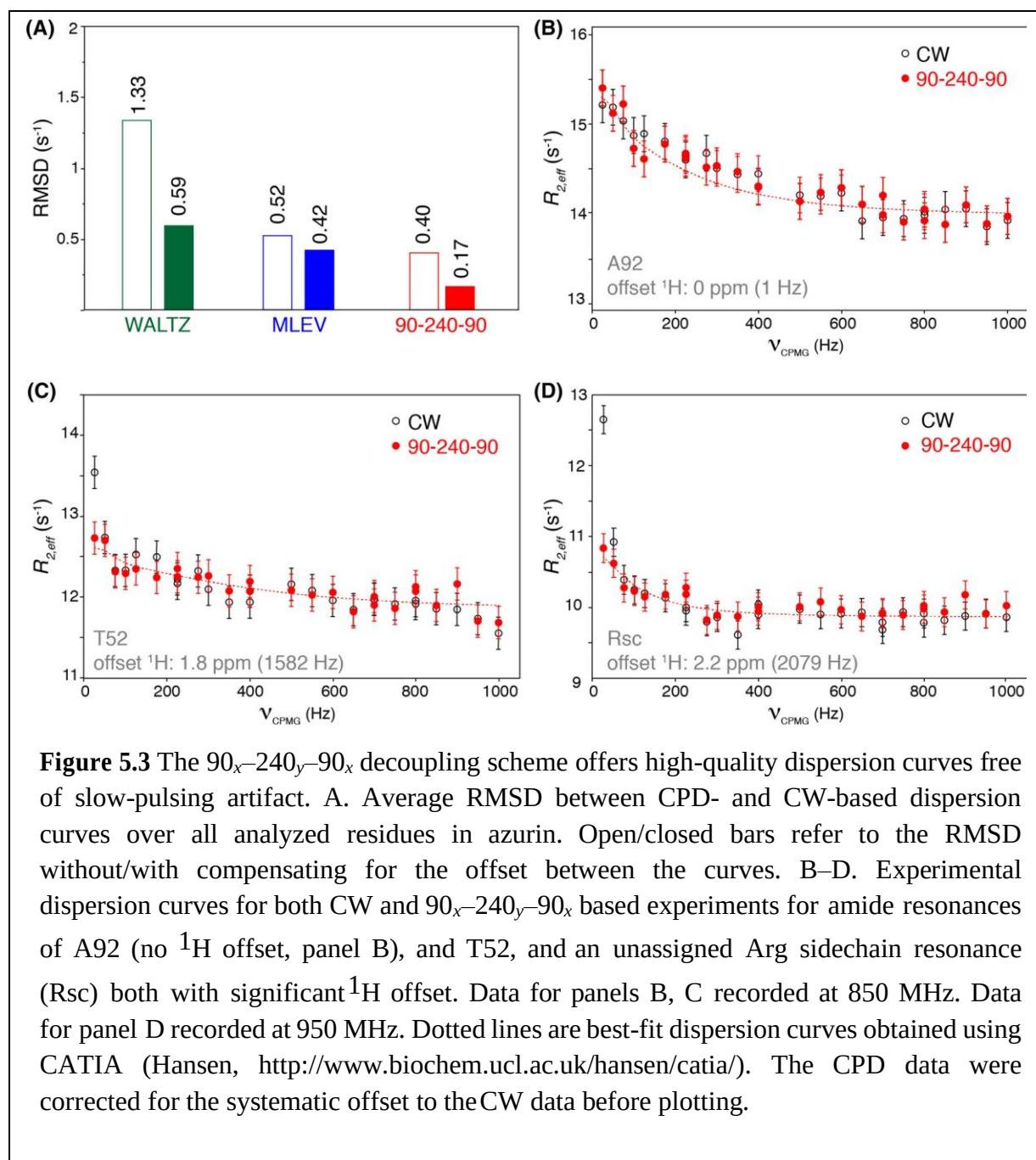


Figure 5.3 The $90_x-240_y-90_x$ decoupling scheme offers high-quality dispersion curves free of slow-pulsing artifact. A. Average RMSD between CPD- and CW-based dispersion curves over all analyzed residues in azurin. Open/closed bars refer to the RMSD without/with compensating for the offset between the curves. B–D. Experimental dispersion curves for both CW and $90_x-240_y-90_x$ based experiments for amide resonances of A92 (no ^1H offset, panel B), and T52, and an unassigned Arg sidechain resonance (Rsc) both with significant ^1H offset. Data for panels B, C recorded at 850 MHz. Data for panel D recorded at 950 MHz. Dotted lines are best-fit dispersion curves obtained using CATIA (Hansen, <http://www.biochem.ucl.ac.uk/hansen/catia/>). The CPD data were corrected for the systematic offset to the CW data before plotting.

In the original implementation of the in-phase dispersion experiment described by Hansen *et al.*³³, the strength of the decoupling field is matched to the CPMG pulsing rate to avoid the timing mismatch as indicated in Figure 5.2C. In principle, such matching could also be done when using CPD decoupling schemes, which should result in decreased scatter in the dispersion curves. While simulations indeed show such improvement in performance, an experimental test showed a severe increase in scatter, presumably due to a point-to-point variation in the steady state of the

water and aliphatic proton magnetization.

As noted in Figure 5.1, the slow-pulsing artifact will be particularly problematic at high magnetic field strengths. At such high fields, it may be better to use TROSY–CPMG sequences⁵⁵, which do not suffer from the slow-pulsing artifact, even for non-deuterated moderately sized proteins. The relative sensitivity of TROSY and in-phase CPMG experiments is best assessed experimentally as it not only depends on magnetic field strength but also on protein size, labeling pattern, and temperature. Next to the absolute sensitivity, one may also consider that lower ¹⁵N relaxation rates during the CPMG period allow the use of longer CPMG delays, increasing the sensitivity to slow motions⁵⁵, as well as spectral quality of TROSY spectra (reduced overlap vs. presence of anti-TROSY lines). Additionally, in case data at lower field strength have been recorded using the in-phase CPMG experiment it may be necessary to record these at high fields too.

In conclusion, we show here that the use of broadband ¹H decoupling, in particular using the 90_x–240_y–90_x sequence, is a viable and attractive option for recording in-phase ¹⁵N relaxation dispersion data. This option is particularly relevant when the protein spectrum contains resonances far from center. It offers artifact-free dispersion profiles without the need for recording data in multiple sets or the need for eliminating of data points, all without compromising data quality.

Materials and methods

NMR samples. NMR experiments were recorded on a sample of 2.5 mM uniformly ¹⁵N/¹³C-labelled Cu(II) azurin in 25 mM potassium phosphate buffer at pH 5.49 with 5% D₂O. Labelled azurin was produced and purified according to a previously published protocol with modifications for incorporating ¹³C-glucose and ¹⁵N-ammoniumchloride³⁷.

NMR experiments. Relaxation dispersion experiments, using the ST–CW–CPMG sequence, were recorded at 298 K on Bruker Advance III HD spectrometers operating at 850 and 950 MHz ¹H Larmor frequency and equipped with TCI cryoprobes. The constant-time CPMG relaxation delay (*T*_{relax}) was set to 40 ms with *v*_{CPMG} set to 25, 50, 75, 100, 125, 175, 225 (2×), 275, 300, 350, 400 (2×), 500, 550, 600, 650, 700 (2×), 750, 800 (2×), 850, 900, 950 and 1000 Hz respectively, run

in an interleaved manner. Duplicates were used to estimate the error in $R_{2,eff}$. The errors were set to 0.2 s^{-1} at minimum. The pulse length of the ^{15}N refocusing pulses in the CPMG train was $90 \mu\text{s}$. For ^1H decoupling, either CW decoupling or a CPD-scheme (GARP, DIPSI, MLEV16, WALTZ16, $90_x-240_y-90_x$) was used. This was implemented by changing the “cw:f1” statement in the pulse program to read “cpds1:f1” (pulse program available upon request). In either case, the decoupling field strength was 14.7 kHz ($17 \mu\text{s}$ ^1H 90° pulse), applied at 8.2 ppm ^1H offset. A total of $3072/120$ points were acquired in the $^1\text{H}/^{15}\text{N}$ dimension with an acquisition time of $90/27.85 \text{ ms}$ and a relaxation delay of 2 s and 4 scans per FID. A reference spectrum, without the relaxation delay, was also recorded. NMR data were processed with NMR Pipe³⁸, using linear prediction in the ^{15}N dimension and Lorentz-to-Gauss window functions. Peak volumes were obtained by peak fitting using FuDa (Hansen, <http://www.biochem.ucl.ac.uk/hansen/fuda/>), and subsequently converted into effective relaxation rates via $R_{2,eff}(v_{\text{CPMG}}) = -1/T_{\text{relax}} \cdot \ln(I(v_{\text{CPMG}})/I_0)$, where I_0 is the peak intensity in a reference spectrum recorded without the relaxation delay T_{relax} . The $R_{2,eff}$ values measured using the ST-CW-CPMG sequence were corrected for R_1 -contribution according to the formula described by Jiang *et al.*³⁴ using an estimate of 0.95 s^{-1} R_1 - and 10.5 s^{-1} for R_2 -contribution for all residues. Dispersion curves obtained with either CW or CPD decoupling were compared by calculating the RSMD between the curves for all residues:

$$RMSD = \sqrt{\frac{1}{N} \sum_{i=1}^N \left(R_{2,eff}^{CPD,i} - R_{2,eff}^{CW,i} \right)^2}$$

where i is the index of a particular v_{CPMG} value and the summation runs over the N recorded points, equal to the number of points per dispersion curve (M) times the number of residues. The systematic difference between the CW or CPD-based dispersion curves was calculated from the average point-by-point difference per residue and is tabulated in Table S5.1. To compensate for these systematic differences, an “ R_2 -offset compensated” RMSD was calculated by replacing the CPD-based $R_{2,eff}$ values with the offset compensated values:

$$R_{2,eff}^{CPD,compensated,i} = R_{2,eff}^{CPD,i} - \frac{1}{M} \sum_{i=1}^M \left(R_{2,eff}^{CPD,i} - R_{2,eff}^{CW,i} \right)$$

Simulation of ^{15}N CW–CPMG dispersion profiles. To evaluate the magnitude of the slow-pulsing artifact in relaxation dispersion profiles, numerical simulations of a two-spin ^1H – ^{15}N system were carried out, assuming a non-deuterated protein. The evolution of magnetization in this spin system was calculated for the CPMG part of the CPMG–CW and CPMG–ST–CW sequence, including the flanking ^{15}N 90° pulses. Simulations in the absence of exchange and neglecting pulse imperfections were performed in operator space by solving the complete homogeneous master equation as described by Allard *et al.*³⁹ and Helgstrand *et al.*⁴⁰ using the open source computing language GNU Octave (<http://www.gnu.org/software/octave/>)⁴¹. All simulation used the parameters detailed below unless noted otherwise. The ^{15}N spin was assumed to be on resonance.

The magnetic field strength was set to 19.9 T, corresponding to ^1H Larmor frequency of 850 MHz. Relaxation rates were calculated using overall rotational correlation time τ_c of 9 ns, a value of 0.85 for the squared generalized order parameter, 100 ps for the correlation time for internal motions, and $-172/+10$ ppm for the $^{15}\text{N}/^1\text{H}$ chemical shift anisotropy. Relaxation due to neighboring protons was included as described in ref Allard *et al.*³⁹ by including a virtual proton at 1.85 Å, resulting in R_2 values of in-phase and anti-phase ^{15}N magnetizations of 13.6 and 26.7 s^{-1} respectively. Dispersion experiments were simulated with T_{relax} set to 40 ms, and ν_{CPMG} values ranging from 25 to 1000 Hz, the ^{15}N 180° refocusing pulse was set to 90 μs , ^1H CW decoupling field strength was set to 14.7 kHz (17 μs ^1H 90°).

Supplementary

Table S5.1 $R_{2,\text{eff}}$ offset between CPD and CW-derived dispersion curves^a.

<i>CPD scheme</i>	<i>average (s^{-1})</i>	<i>range (s^{-1})</i>	<i>90% limit (s^{-1})^b</i>
$90_x-240_y-90_x$	-0.27	-1.13 – 0.74	0.57
<i>MLEV</i>	-1.24	-6.77 – 1.06	2.92
<i>WALTZ</i>	-0.85	-4.44 – 1.15	1.80

^a data recorded with offset of decoupling field centered at 8.2 ppm. Negative offset values indicate that CPD-derived $R_{2,\text{eff}}$ values are lower than in the CW-based experiment.

^b absolute values.

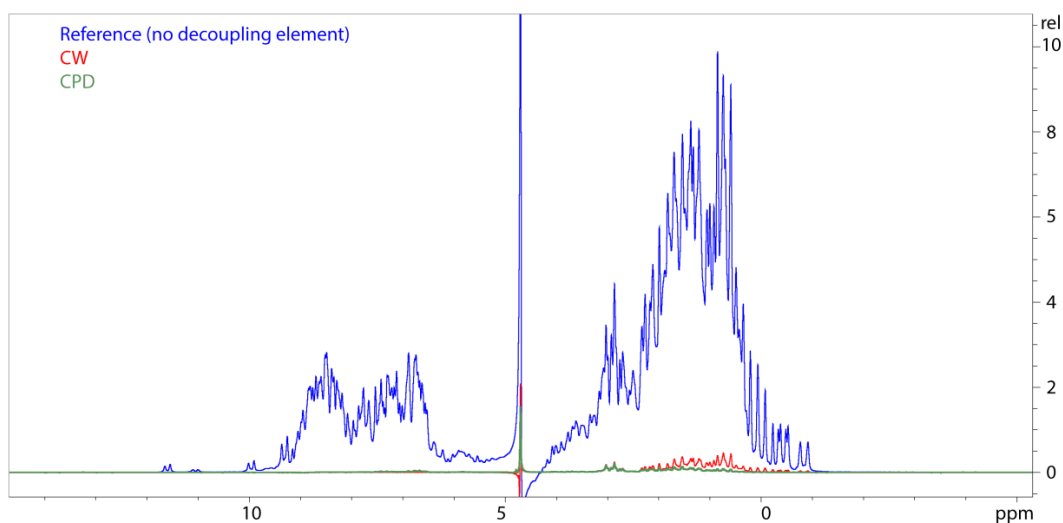


Figure S5.1 1D- ^1H NMR spectra of azurin to study the effect on the reference experiment where the decoupling block is placed before the recycle delay. A recycle delay of 40 ms was used for obtaining data for CW and CPD elements while keeping the decoupling offset at 8.2 ppm and the decoupling power set at 3.5 W. The reference spectrum does not contain any decoupling block. Both CW and CPD decouplers suppress the water and aliphatic protons.

References

- (1) Frauenfelder, H., Sligar, S. G., and Wolynes, P. G. (1991) The energy landscapes and motions of proteins. *Science* (80-.). 254, 1598–1603.
- (2) Wolynes, P. G., Karplus, M., Dobson, C. M., Zewail, A. H., Clarke, J., and Schön, J. C. (2005) Energy landscapes and solved protein-folding problems. *Philos. Trans. R. Soc. A Math. Phys. Eng. Sci.* 363, 453–467.
- (3) Henzler-Wildman, K., and Kern, D. (2007, December) Dynamic personalities of proteins. *Nature*. Nature Publishing Group.
- (4) HAMMES, G. G. (1964) Mechanism of enzyme catalysis. *Nature* 204, 342–343.
- (5) Eisenmesser, E. Z., Millet, O., Labeikovsky, W., Korzhnev, D. M., Wolf-Watz, M., Bosco, D. A., Skalicky, J. J., Kay, L. E., and Kern, D. (2005) Intrinsic dynamics of an enzyme underlies catalysis. *Nature* 438, 117–121.
- (6) Henzler-Wildman, K. A., Thai, V., Lei, M., Ott, M., Wolf-Watz, M., Fenn, T., Pozharski, E., Wilson, M. A., Petsko, G. A., Karplus, M., Hübner, C. G., and Kern, D. (2007) Intrinsic motions along an enzymatic reaction trajectory. *Nature* 450, 838.
- (7) Palmer, A. G. (2015) Enzyme dynamics from NMR spectroscopy. *Acc. Chem. Res.* 48, 457–465.
- (8) Kim, T. H., Mehrabi, P., Ren, Z., Sljoka, A., Ing, C., Bezginov, A., Ye, L., Pomès, R., Prosser, R. S., and Pai, E. F. (2017) The role of dimer asymmetry and protomer dynamics in enzyme catalysis. *Science* (80-.). 355.
- (9) Sugase, K., Dyson, H. J., and Wright, P. E. (2007) Mechanism of coupled folding and binding of an intrinsically disordered protein. *Nature* 447, 1021.
- (10) Schneider, R., Maurin, D., Communie, G., Kragelj, J., Hansen, D. F., Ruigrok, R. W. H., Jensen, M. R., and Blackledge, M. (2015) Visualizing the molecular recognition trajectory of an intrinsically disordered protein using multinuclear relaxation dispersion NMR. *J. Am. Chem. Soc.* 137, 1220–1229.
- (11) Pratihar, S., Sabo, T. M., Ban, D., Fenwick, R. B., Becker, S., Salvatella, X., Griesinger, C.,

and Lee, D. (2016) Kinetics of the Antibody Recognition Site in the Third IgG-Binding Domain of Protein G. *Angew. Chemie - Int. Ed.* 55, 9567–9570.

(12) Xiao, T., Fan, J., Zhou, H., Lin, Q., and Yang, D. (2016) Local unfolding of fatty acid binding protein to allow ligand entry for binding. *Angew. Chemie Int. Ed.* 55, 6869–6872.

(13) Zhao, B., Guffy, S. L., Williams, B., and Zhang, Q. (2017) An excited state underlies gene regulation of a transcriptional riboswitch. *Nat. Chem. Biol.* 13, 968–974.

(14) Delaforge, E., Kragelj, J., Tengo, L., Palencia, A., Milles, S., Bouvignies, G., Salvi, N., Blackledge, M., and Jensen, M. R. (2018) Deciphering the dynamic interaction profile of an intrinsically disordered protein by NMR exchange spectroscopy. *J. Am. Chem. Soc.* 140, 1148–1158.

(15) Korzhnev, D. M., Religa, T. L., Banachewicz, W., Fersht, A. R., and Kay, L. E. (2010) A transient and low-populated protein-folding intermediate at atomic resolution. *Science* (80-.). 329, 1312 LP – 1316.

(16) Neudecker, P., Robustelli, P., Cavalli, A., Walsh, P., Lundström, P., Zarrine-Afsar, A., Sharpe, S., Vendruscolo, M., and Kay, L. E. (2012) Structure of an intermediate state in protein folding and aggregation. *Science* (80-.). 336, 362 LP – 366.

(17) Kimsey, I. J., Petzold, K., Sathyamoorthy, B., Stein, Z. W., and Al-Hashimi, H. M. (2015) Visualizing transient Watson-Crick-like mispairs in DNA and RNA duplexes. *Nature* 519, 315–320.

(18) Libich, D. S., Tugarinov, V., and Clore, G. M. (2015) Intrinsic unfoldase/foldase activity of the chaperonin GroEL directly demonstrated using multinuclear relaxation-based NMR. *Proc. Natl. Acad. Sci. U. S. A.* 112, 8817–8823.

(19) Franco, R., Gil-Caballero, S., Ayala, I., Favier, A., and Brutscher, B. (2017) Probing conformational exchange dynamics in a short-lived protein folding intermediate by real-time relaxation–dispersion NMR. *J. Am. Chem. Soc.* 139, 1065–1068.

(20) Culik, R. M., Sekhar, A., Nagesh, J., Deol, H., Rumfeldt, J. A. O., Meiering, E. M., and Kay, L. E. (2018) Effects of maturation on the conformational free-energy landscape of SOD1. *Proc. Natl. Acad. Sci. U. S. A.* 115, E2546–E2555.

- (21) Akke, M., and Palmer, A. G. (1996) Monitoring macromolecular motions on microsecond to millisecond time scales by $R_{1\rho}$ – R_1 constant relaxation time NMR spectroscopy. *J. Am. Chem. Soc.* **118**, 911–912.
- (22) Fawzi, N. L., Ying, J., Torchia, D. A., and Clore, G. M. (2010) Kinetics of amyloid β monomer-to-oligomer exchange by NMR relaxation. *J. Am. Chem. Soc.* **132**, 9948–9951.
- (23) Kovermann, M., Rogne, P., and Wolf-Watz, M. (2016) Protein dynamics and function from solution state NMR spectroscopy. *Q. Rev. Biophys.* **49**, e6.
- (24) Palmer, A. G., Kroenke, C. D., and Loria, J. P. (2001) Nuclear magnetic resonance methods for quantifying microsecond-to-millisecond motions in biological macromolecules, in *Methods in Enzymology*, pp 204–238. Academic Press Inc.
- (25) Vallurupalli, P., Bouvignies, G., and Kay, L. E. (2012) Studying “invisible” excited protein states in slow exchange with a major state conformation. *J. Am. Chem. Soc.* **134**, 8148–8161.
- (26) Vallurupalli, P., Sekhar, A., Yuwen, T., and Kay, L. E. (2017) Probing conformational dynamics in biomolecules via chemical exchange saturation transfer: a primer. *J. Biomol. NMR* **67**, 243–271.
- (27) Sauerwein, A. C., and Flemming Hansen, D. (2015) Relaxation dispersion NMR spectroscopy, in *Protein NMR: Modern Techniques and Biomedical Applications*, pp 75–132. Springer International Publishing.
- (28) Xue, Y., Kellogg, D., Kimsey, I. J., Sathyamoorthy, B., Stein, Z. W., McBairty, M., and Al-Hashimi, H. M. (2015) Characterizing RNA excited states using NMR relaxation dispersion, in *Methods in Enzymology*, pp 39–73. Academic Press Inc.
- (29) Lisi, G. P., and Loria, J. P. (2016, February) Using NMR spectroscopy to elucidate the role of molecular motions in enzyme function. *Prog. Nucl. Magn. Reson. Spectrosc.* Elsevier B.V.
- (30) Massi, F., and Peng, J. W. (2018) Characterizing protein dynamics with NMR $R_{1\rho}$ relaxation experiments, in *Methods in Molecular Biology*, pp 205–221. Humana Press Inc.
- (31) Gopalan, A. B., Hansen, D. F., and Vallurupalli, P. (2018) CPMG experiments for protein minor conformer structure determination, in *Methods in Molecular Biology*, pp 223–242.

Humana Press Inc.

- (32) Loria, J. P., Rance, M., and Palmer, A. G. (1999, March) A relaxation-compensated Carr-Purcell-Meiboom-Gill sequence for characterizing chemical exchange by NMR spectroscopy [13]. *J. Am. Chem. Soc.* American Chemical Society .
- (33) Hansen, D. F., Vallurupalli, P., and Kay, L. E. (2008) An improved ^{15}N relaxation dispersion experiment for the measurement of millisecond time-scale dynamics in proteins. *J. Phys. Chem. B* 112, 5898–5904.
- (34) Jiang, B., Yu, B., Zhang, X., Liu, M., and Yang, D. (2015) A ^{15}N CPMG relaxation dispersion experiment more resistant to resonance offset and pulse imperfection. *J. Magn. Reson.* 257, 1–7.
- (35) Yip, G. N. B., and Zuiderweg, E. R. P. (2004) A phase cycle scheme that significantly suppresses offset-dependent artifacts in the R2-CPMG ^{15}N relaxation experiment. *J. Magn. Reson.* 171, 25–36.
- (36) Chakrabarti, K. S., Ban, D., Pratihar, S., Reddy, J. G., Becker, S., Griesinger, C., and Lee, D. (2016) High-power ^1H composite pulse decoupling provides artifact free exchange-mediated saturation transfer (EST) experiments. *J. Magn. Reson.* 269, 65–69.
- (37) Karlsson, B. G., Pascher, T., Nordling, M., Arvidsson, R. H. A., and Lundberg, L. G. (1989) Expression of the blue copper protein azurin from *Pseudomonas aeruginosa* in *Escherichia coli*. *FEBS Lett.* 246, 211–217.
- (38) Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) NMRPipe: A multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* 6, 277–293.
- (39) Allard, P., Helgstrand, M., and Härd, T. (1998) The Complete Homogeneous Master Equation for a Heteronuclear Two-Spin System in the Basis of Cartesian Product Operators. *J. Magn. Reson.* 134, 7–16.
- (40) Helgstrand, M., Hard, T., and Allard, P. (2000) Simulations of NMR pulse sequences during equilibrium and non-equilibrium chemical exchange. *J. Biomol. NMR* 18, 49–63.

- (41) GNU Octave Manual Version 3 | Perceiving Systems - Max Planck Institute for Intelligent Systems.
- (42) Palmer, A. G., Skelton, N. J., Chazin, W. J., Wright, P. E., and Rance, M. (1992) Suppression of the effects of cross-correlation between dipolar and anisotropic chemical shift relaxation mechanisms in the measurement of spin-spin relaxation rates. *Mol. Phys.* 75, 699–711.
- (43) Hansen, D. F., Vallurupalli, P., Lundström, P., Neudecker, P., and Kay, L. E. (2008) Probing chemical shifts of invisible states of proteins with relaxation dispersion NMR spectroscopy: how well can we do? *J. Am. Chem. Soc.* 130, 2667–2675.
- (44) Adman, E. T. (1991, January) Copper protein structures. *Adv. Protein Chem.* Academic Press Inc.
- (45) Korzhnev, D. M. (2003) NMR detection of multiple transitions to low-populated states in azurin. *Protein Sci.* 12, 56–65.
- (46) Shaka, A. J., and Keeler, J. (1987, January) Broadband spin decoupling in isotropic-liquids. *Prog. Nucl. Magn. Reson. Spectrosc.* Pergamon.
- (47) Shaka, A. J., Barker, P. B., and Freeman, R. (1985) Computer-optimized decoupling scheme for wideband applications and low-level operation. *J. Magn. Reson.* 64, 547–552.
- (48) Shaka, A. J., Lee, C. J., and Pines, A. (1988) Iterative schemes for bilinear operators; application to spin decoupling. *J. Magn. Reson.* 77, 274–293.
- (49) Shaka, A. J., Keeler, J., Frenkiel, T., and Freeman, R. (1983) An improved sequence for broadband decoupling: WALTZ-16. *J. Magn. Reson.* 52, 335–338.
- (50) Levitt, M. H., and Freeman, R. (1981) Composite pulse decoupling. *J. Magn. Reson.* 43, 502–507.
- (51) Levitt, M. H., Freeman, R., and Frenkiel, T. (1982) Supercycles for broadband heteronuclear decoupling. *J. Magn. Reson.* 50, 157–160.
- (52) Levitt, M. H., Freeman, R., and Frenkiel, T. (1982) Broadband heteronuclear decoupling. *J. Magn. Reson.* 47, 328–330.
- (53) Levitt, M. H. (1982) Symmetrical composite pulse sequences for NMR population

inversion. II. Compensation of resonance offset. *J. Magn. Reson.* 50, 95–110.

(54) Hiller, S., Wider, G., Etezady-Esfarjani, T., Horst, R., and Wüthrich, K. (2005) Managing the solvent water polarization to obtain improved NMR spectra of large molecular structures. *J. Biomol. NMR* 32, 61–70.

(55) Loria, J. P., Rance, M., and Palmer, A. G. (1999) A TROSY CPMG sequence for characterizing chemical exchange in large proteins. *J. Biomol. NMR* 15, 151–155.