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Supporting Information

Measuring Diffusion Constants of Invisible Protein Conformers by Triple-Quantum ^{1}H CPMG Relaxation Dispersion

*Tairan Yuwen, Ashok Sekhar, Andrew J. Baldwin, Pramodh Vallurupalli, and Lewis E. Kay**

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Supporting Information

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Materials and Methods

Sample Preparation

An NMR sample of [U-^{15}N; U-^{2}H; Ileδ1-^{13}CH$_3$; Leu,Val-^{13}CH$_3$/^{12}CD$_3$]-labeled G48A Fyn SH3 was prepared as described previously.[1] The sample concentration was 1.1 mM in a buffer containing 50 mM sodium phosphate, 0.2 mM EDTA, 0.05% NaN_3, pH 7.0, 90% H_2O/10% D_2O.

NMR Spectroscopy

All NMR experiments were carried out at 25 °C on Bruker AVANCE III spectrometers equipped with cryogenically cooled probes with *z*- (800 MHz) or triple-axis (600 MHz) pulsed field gradients. Methyl 3Q-CPMG relaxation dispersion experiments were recorded using a constant-time CPMG element[2,3] with T_{relax} = 40 ms using the pulse scheme of Figure 2. Experiments were recorded as pseudo-3D datasets by varying the number of CPMG pulses N during T_{relax} in a series of 2D planes (1 value of N per plane). A series of 2D planes was obtained with ν_{CPMG} varying between 25 Hz and 1000 Hz so that totally 29 planes were recorded, including duplicates for error analysis.[4] CPMG experiments with the gradient strength g_3 set to 0 G/cm were performed on both 600 and 800 MHz spectrometers in order to determine (p_E, k_{ex}) accurately, while experiments with g_3 = 30.6 G/cm were only carried out at 800 MHz. CPMG datasets with g_3 set to 0 or 30.6 G/cm were recorded with 6 or 24 scans/FID, a relaxation delay of 2.5 s and (768, 48) complex points in (t_2, t_1) to give net acquisition times of ~25 or ~100 min/spectrum, and thus total measurement times of ~12 or ~48 h, respectively. The duration of each g_3 gradient is 1 ms (see Figures 2 and S1), which leads to ~80% signal decay during T_{relax} = 40 ms for G48A Fyn SH3. The value used for g_3 is slightly larger than optimal (25 G/cm) in terms of signal-to-noise as discussed in the main text.

Data Analysis

All NMR spectra were processed and analyzed using the *NMRPipe* suite of programs.[5] Effective transverse relaxation rates, $R_{2,eff}$, were calculated based on peak intensities according to the relation $R_{2,eff}(\nu_{CPMG}) = -\ln(I(\nu_{CPMG})/I_0)/T_{relax}$, where I_0 is the peak intensity in a reference spectrum recorded without the relaxation delay, T_{relax}.[2] Fitting of cross-peaks in CPMG data sets was carried out using the software package *ChemEx* (https://github.com/gbouvignies/chemex); a separate module is required for fitting the 3Q ^{1}H relaxation dispersion data with the encode/decode gradients that is available upon request. Only residues with dispersion profiles such that $R_{ex} = R_{2,eff}(\nu_{CPMG} = 25\text{ Hz}) - R_{2,eff}(\nu_{CPMG} = 1000\text{ Hz}) > 2\text{ s}^{-1}$ at 800 MHz, $g_3 = 0$ G/cm were included in the CPMG data analysis. Exchange parameters were extracted from fits of dispersion data to a two-site exchange model using the Bloch–McConnell equations[6] and assuming that $\Delta R_2 = 0$ (see below).

As discussed previously,[7] methyl ^{1}H 3Q coherences evolve as a result of the one-bond scalar coupling to the attached ^{13}C spin during the CPMG element, interconverting between in-phase and anti-phase components that relax differently.[8] The interconversion of these elements during intervals between ^{1}H CPMG refocusing pulses can lead to small contributions to $R_{2,eff}$ values in dispersion profiles. This effect can be accounted for during data fitting by including an additional fitting parameter or by fixing the differential decay rate to that measured in separate experiments.[7] Alternatively, a modified pulse scheme that generates equal amounts of in-phase and anti-phase 3Q coherences at the start of the CPMG period can be employed (Figure S1). Simulations indicate that for differential relaxation rates less than 5 s^{-1} (which is, in general, always the case), this approach eliminates the (small) modulation of the CPMG dispersion profiles that would otherwise result.

Discussion

Further Examination of the Bloch–McConnell Equations, Including Diffusion

In the main text we provide a qualitative explanation of the origin of Eq (3); herein a more detailed derivation is given for completeness. Modifications to the original Bloch equations to account for diffusion and chemical exchange have been presented by Torrey[9] and McConnell[6] respectively,

$$\begin{aligned}\frac{\partial m_+^G(t,z)}{\partial t} &= -i\omega_G m_+^G(t,z) + D^G\frac{\partial^2 m_+^G(t,z)}{\partial z^2} - R_2^G m_+^G(t,z) - k_{GE}m_+^G(t,z) + k_{EG}m_+^E(t,z),\\ \frac{\partial m_+^E(t,z)}{\partial t} &= -i\omega_E m_+^E(t,z) + D^E\frac{\partial^2 m_+^E(t,z)}{\partial z^2} - R_2^E m_+^E(t,z) + k_{GE}m_+^G(t,z) - k_{EG}m_+^E(t,z)\end{aligned} \tag{S1}$$

where $m_+(t,z) = m_x(t,z) + im_y(t,z)$ is transverse magnetization, $m_j(t,z)$ is its j component and $i = \sqrt{-1}$. Note that magnetization is a function both of time and of the spatial coordinate z since z-pulsed field gradients are considered. Eq (S1) is complicated by the second derivate but can be solved straightforwardly in Fourier space as discussed by Johnson.[10] Defining $m_+(t,z)$ and $M_+(t,K)$ as Fourier pairs so that

$$\begin{aligned}M_+(t,K) &= \int_{-\infty}^{\infty} m_+(t,z)e^{-iKz}dz = \mathscr{F}\{m_+(t,z)\}\\ m_+(t,z) &= \frac{1}{2\pi}\int_{-\infty}^{\infty} M_+(t,K)e^{iKz}dK = \mathscr{F}^{-1}\{M_+(t,K)\}\end{aligned} \tag{S2}$$

it follows immediately that

$$\frac{\partial M_+(t,K)}{\partial t} = \int_{-\infty}^{\infty}\frac{\partial m_+(t,z)}{\partial t}e^{-iKz}dz \tag{S3}$$

so that $\dfrac{\partial m_+(t,z)}{\partial t}$ and $\dfrac{\partial M_+(t,K)}{\partial t}$ are Fourier pairs, that is $\dfrac{\partial M_+(t,K)}{\partial t} = \mathscr{F}\left\{\dfrac{\partial m_+(t,z)}{\partial t}\right\}$, where $\mathscr{F}$ denotes the Fourier transform. In a similar manner starting from Eq (S2) it follows that

$$\frac{\partial^2 m_+(t,z)}{\partial z^2} = -\frac{1}{2\pi}\int_{-\infty}^{\infty} K^2 M_+(t,K)e^{iKz}dK \tag{S4}$$

so that

$$\frac{\partial^2 m_+(t,z)}{\partial z^2} = -\mathscr{F}^{-1}\{K^2 M_+(t,K)\} \;\Rightarrow\; \mathscr{F}\left\{\frac{\partial^2 m_+(t,z)}{\partial z^2}\right\} = -K^2 M_+(t,K). \quad \text{(S5)}$$

Taking the Fourier transform of both sides of Eq (S1) then gives

$$\begin{aligned}
\frac{\partial M_+^G(t,K)}{\partial t} &= -i\omega_G M_+^G(t,K) - K^2 D^G M_+^G(t,K) - R_2^G M_+^G(t,K) - k_{GE} M_+^G(t,K) + k_{EG} M_+^E(t,K), \\
\frac{\partial M_+^E(t,K)}{\partial t} &= -i\omega_E M_+^E(t,K) - K^2 D^E M_+^E(t,K) - R_2^E M_+^E(t,K) + k_{GE} M_+^G(t,K) - k_{EG} M_+^E(t,K)
\end{aligned} \quad \text{(S6)}$$

which is Eq (3) of the main text. The effect of the Fourier transform is to evoke a coordinate transformation (from z to K) to generate a set of coupled equations that are analogous to the Bloch–McConnell equations in the absence of diffusion (with R_2 replaced by $R_2 + K^2 D$). Eq (S6) can be readily solved in the usual manner.

Size Limitations of the Methodology

As described in the text we have used the ^{1}H 3Q-CPMG pulse scheme for measurement of translational diffusion constants of rare protein states because of the high sensitivity of 3Q coherences to pulsed field gradients, scaling with a factor of 3 relative to SQ magnetization. The effects of diffusion, and hence K^2 in Eqs (S3–S6), are scaled 9-fold.[11–14] Intrinsic transverse relaxation rates of ^{1}H 3Q coherences in methyl groups are, however, significantly larger than the corresponding slowly-relaxing SQ coherences, even though both sets of coherences are immune to intra-methyl ^{1}H–^{1}H dipolar interactions in the limit of slowly tumbling protein molecules.[15,16] The differential relaxation is largely the result of contributions from intra-methyl ^{1}H–^{13}C dipolar relaxation and relaxation with proton spins external to the methyl group in question. The latter effects can be considerable even in cases of highly deuterated, methyl protonated proteins, such as used here. Average methyl ^{1}H R_2^{3Q} and ^{1}H $R_2^{SQ_{slow}}$ relaxation rates for T4 lysozyme (T4L) at 15 °C (18 kDa, estimated rotational correlation time of 14.3 ns) have been measured to be ~60 s^{-1} and ~12.5 s^{-1}, respectively.[17] It is expected that the increased relaxation baseline for the 3Q experiment will limit applications to pro-

teins smaller than approximately 20–30 kDa, although it is difficult to estimate the exact molecular weight cutoff for diffusion applications, as this will depend on spectral quality and on the sizes of ΔD and p_E. In this regard, it is worth noting that high quality ^{1}H 3Q CPMG dispersion profiles were recorded for T4L at 15 °C, despite the increased intrinsic transverse relaxation rates for ^{1}H 3Q coherences. In principle, it is, of course, possible to design a diffusion-SQ based CPMG pulse sequence, however encode/decode gradient strengths and durations and likely also T_{relax} would have to increase to compensate for the smaller gradient effects associated with SQ magnetization.

A second point of note is that the application of encode/decode gradients, g, does lead to large attenuations in signal intensities or, equivalently, to large increases in effective relaxation rates, $R_{2,K}$ (see text). As described in the text, there is an optimal size for g, as on one hand larger g values increase $\Delta\Delta R_{2,eff} \propto K^2\Delta D$, yet they also lead to a decrease in spectral intensity. Optimal K values can be estimated from D^G and hence the increase in $R_{2,K}$ can be tuned by the experimentalist to maximize the quality of the diffusion measurements. To first order this occurs when $K^2D^GT_{relax} = 1$ (see text), independent of molecular weight. Assuming a fixed T_{relax} value then the contribution to $R_{2,K}$ from g, K^2D, will also be molecular weight independent. The concomitant increase in intrinsic relaxation rates with size, proportional to molecular weight, is the major factor in limiting the sizes of molecules that can be studied with this experiment.

Influence of ΔR_2 on Extracted D^E Values

As described above the CPMG data were fit under the assumption that ΔR_2 = 0. In order to evaluate the influence that this might have on the extracted diffusion values for the excited state we have refit our data assuming $\Delta R_2 = -R_2^G$ ($R_2^E = 0$) and $\Delta R_2 = R_2^G$ ($R_2^E = 2R_2^G$). As expected, values of p_E and k_{ex} did vary in the predicted way[18] from ($7.6 \pm 0.5\%$, 124.6 ± 9.0 s^{-1}), to ($6.7 \pm 0.3\%$, 126.9 ± 8.7 s^{-1}) and to ($8.3 \pm 0.6\%$, 125.3 ± 9.3 s^{-1}) for $\Delta R_2 = 0$, $-R_2^G$ and R_2^G, respectively, when all 10 dispersions were fit simultaneously. Notably, very little effect on extracted D^G and D^E values was observed, as illustrated in Figure S5, with $D^E = (1.22 \pm 0.04) \times 10^{-6}$ cm^2/s, $(1.23 \pm 0.04) \times$

10^{-6} cm^2/s and $(1.21 \pm 0.04) \times 10^{-6}$ cm^2/s for $\Delta R_2 = 0$, $-R_2^G$ and R_2^G, respectively, while $D^G = (1.57 \pm 0.01) \times 10^{-6}$ cm^2/s was obtained for each case. In general, we recommend fits using a number of different ΔR_2 values (with ΔR_2 constrained in each fit) to assess the interplay between ΔR_2 and ΔD. In the present application ($\Delta R_2 < 0\,\mathrm{s}^{-1}$) the interplay is small. Figure S6 illustrates how exchange parameters (p_E, k_{ex}) and D^E are affected by ΔR_2 more generally.

Supplementary Figures

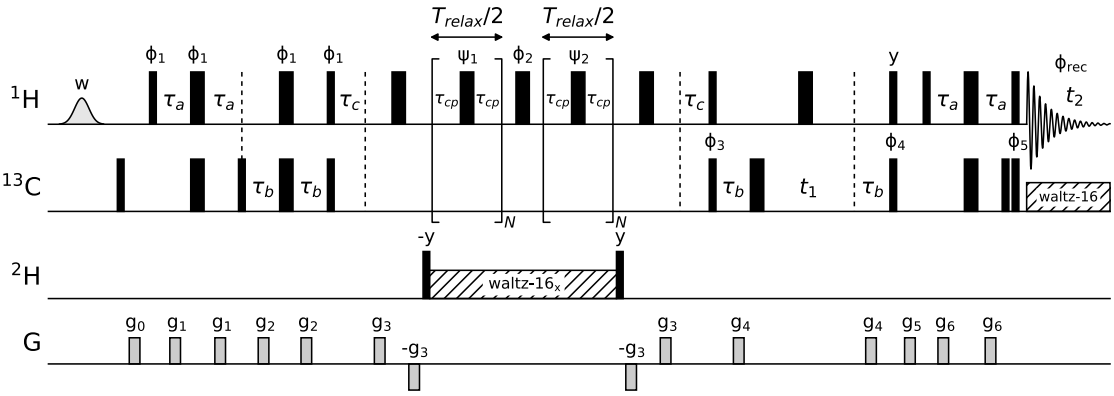

Figure S1. Modified methyl 3Q-CPMG pulse scheme with delay periods (τ_c) to generate equal amounts of IP and AP 3Q coherences in order to suppress differential relaxation effects, as described above. ^{1}H 90° (180°) rectangular pulses, denoted by narrow (wide) black bars, are applied with a 25 kHz field until the final INEPT period where they are at maximum power; ^{13}C pulses are applied at maximum power, while ^{2}H pulses flanking the decoupling element are applied with a 1.7 kHz field. ^{13}C and ^{2}H WALTZ-16 decoupling elements[19] are applied with fields of 2 kHz and 500 Hz, respectively. The delays used are: $\tau_a = 1.8$ ms, $\tau_b = 2$ ms, $\tau_c = 1/(12^1J_{\mathrm{HC}}) = 0.67$ ms, $\tau_{cp} = T_{relax}/(4N)$, N is an integer. The phase cycle is $\phi_1 = (0°, 60°, 120°, 180°, 240°, 300°)$; $\phi_2 = y, -y$; $\phi_3 = 6(x), 6(-x)$; $\phi_4 = x$; $\phi_5 = x, -x$; $\phi_{rec} = 3(x, -x), 3(-x, x)$, with a minimum cycle of 6. The phases ψ_1/ψ_2 are used to implement the XY-16 scheme,[20] as described previously.[7] Quadrature detection in F_1 is achieved by STATES-TPPI of ϕ_4.[21] Gradients are applied with the following durations (ms) and strengths (G/cm): g_0: (1.0, 4), g_1: (0.4, 5), g_2: (0.6, 6), g_3: encode/decode gradients, g_4: (0.3, −5), g_5: (0.5, 10), g_6: (0.3, −8). Further details are provided in Ref. [7].

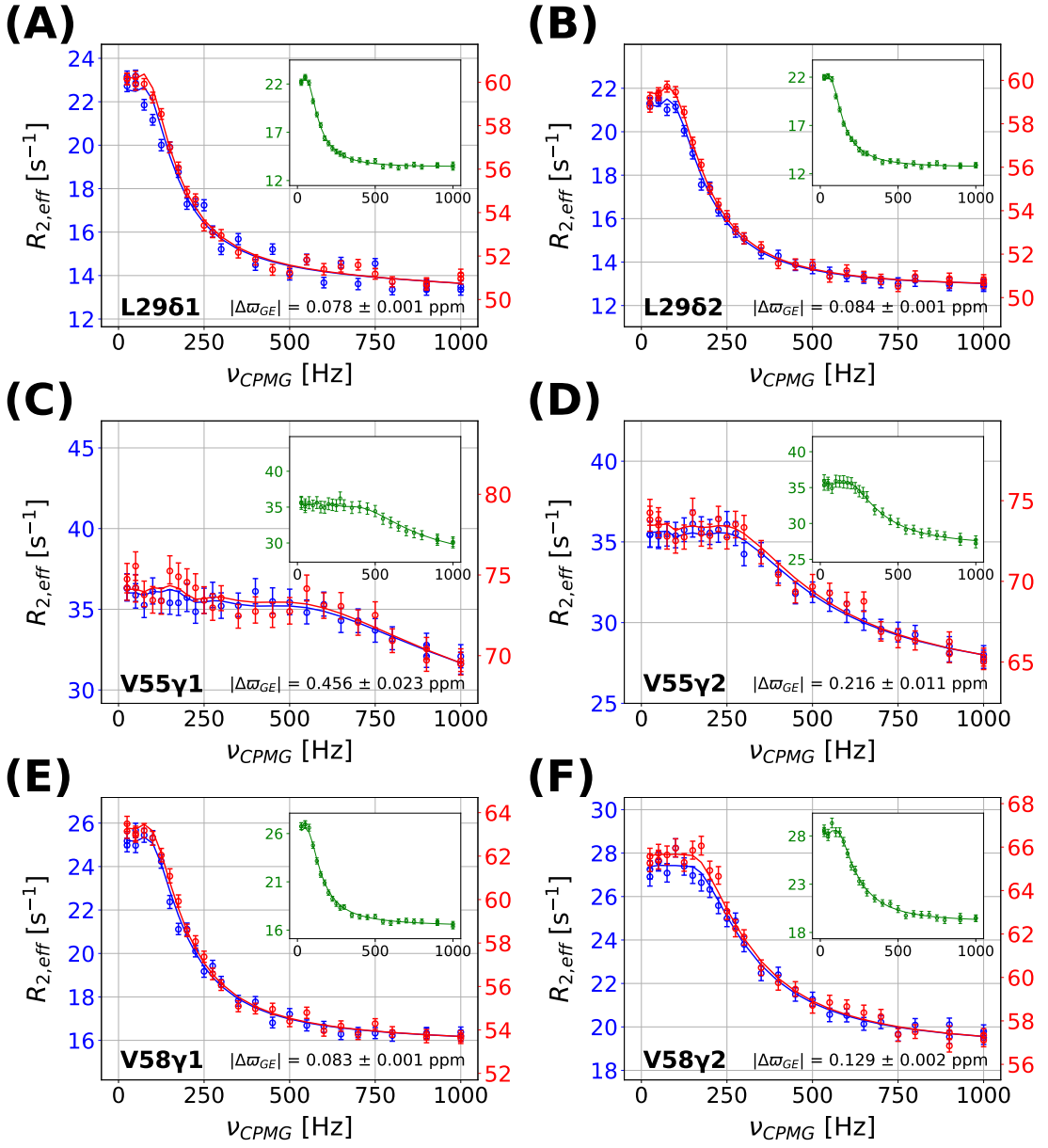

Figure S2. Fits of 3Q-CPMG dispersion profiles for the residues not shown in Figure 3, but included in the global fitting analysis. All details are as in Figure 3.

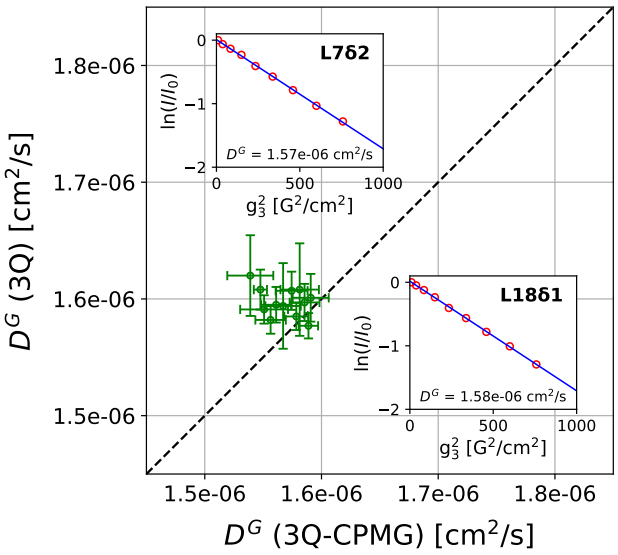

Figure S3. Comparison between per-residue D^G values obtained from fits of CPMG profiles (x-axis), as described in the text, and diffusion constants (y-axis) generated from fits of intensity *vs* g_3^2 profiles (insets) that were measured using the scheme of Figure 2 with $N = 1$. Values of $D^G = (1.60 \pm 0.01) \times 10^{-6}$ cm^2/s and $D^G = (1.57 \pm 0.02) \times 10^{-6}$ cm^2/s ($\pm$ refers to 1 standard deviation of the per-residue measured values) were obtained using the 3Q diffusion approach described previously[14] and the 3Q CPMG method of the present paper, respectively.

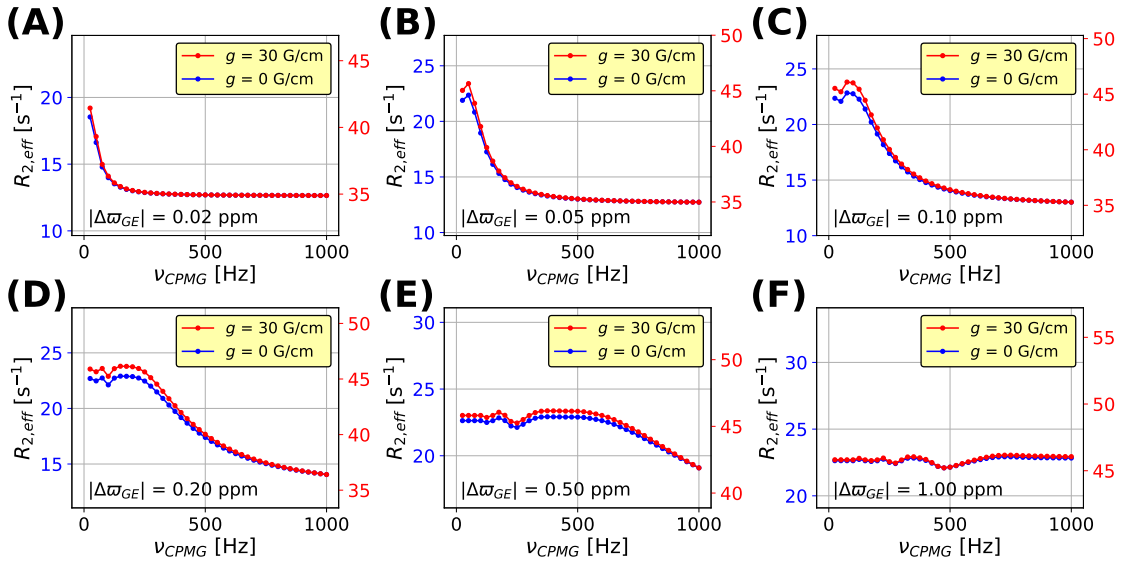

Figure S4. Simulated CPMG dispersion profiles with g_3 = 0 and 30 G/cm as a function of $|\Delta\varpi_{GE}|$. $|\Delta\varpi_{GE}|$ ranging between 0.02–1.0 ppm are used for simulations, along with a static magnetic field of 18.8 T, with all other parameters as in Figure 1. In order to observe meaningful differences between CPMG dispersion profiles recorded with g_3 = 0 and 30 G/cm, and hence to be able to measure accurate diffusion values, ~0.05 ppm $< |\Delta\varpi_{GE}| <$ ~0.50 ppm for the exchange parameters chosen. For very small $\Delta\varpi_{GE}$ values difficulties arise because the effective chemical shift difference is reduced to 0 even for low ν_{CPMG} values, while it is difficult to pulse out the chemical shift difference in cases where $\Delta\varpi_{GE}$ is very large so as to obtain quantifiable differences in the dispersion profiles.

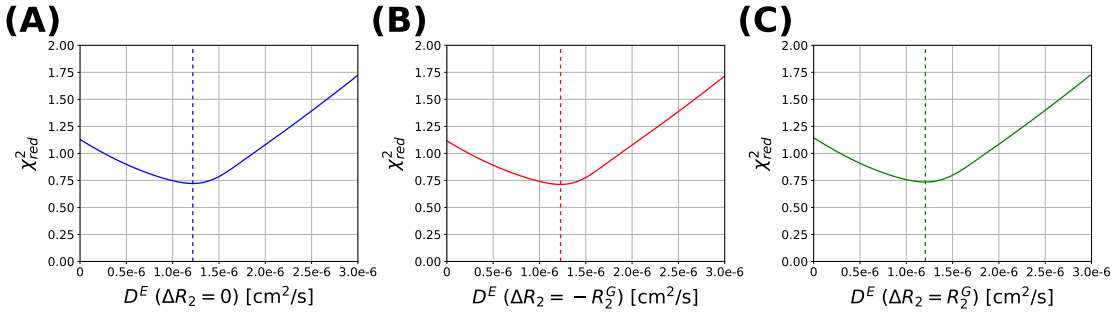

Figure S5. χ^2_{red} surface plots from global fits based on analysis of 10 methyl groups, selected as described in *Data Analysis*, with $\Delta R_2 = R_2^E - R_2^G = 0$ (Panel A), $\Delta R_2 = -R_2^G$ (Panel B) or $\Delta R_2 = R_2^G$ (Panel C). Analyses were carried out using the program *ChemEx*. The dashed line in each panel indicates the position of D^E that gives a minimum in the χ^2_{red} profile for each global fit.

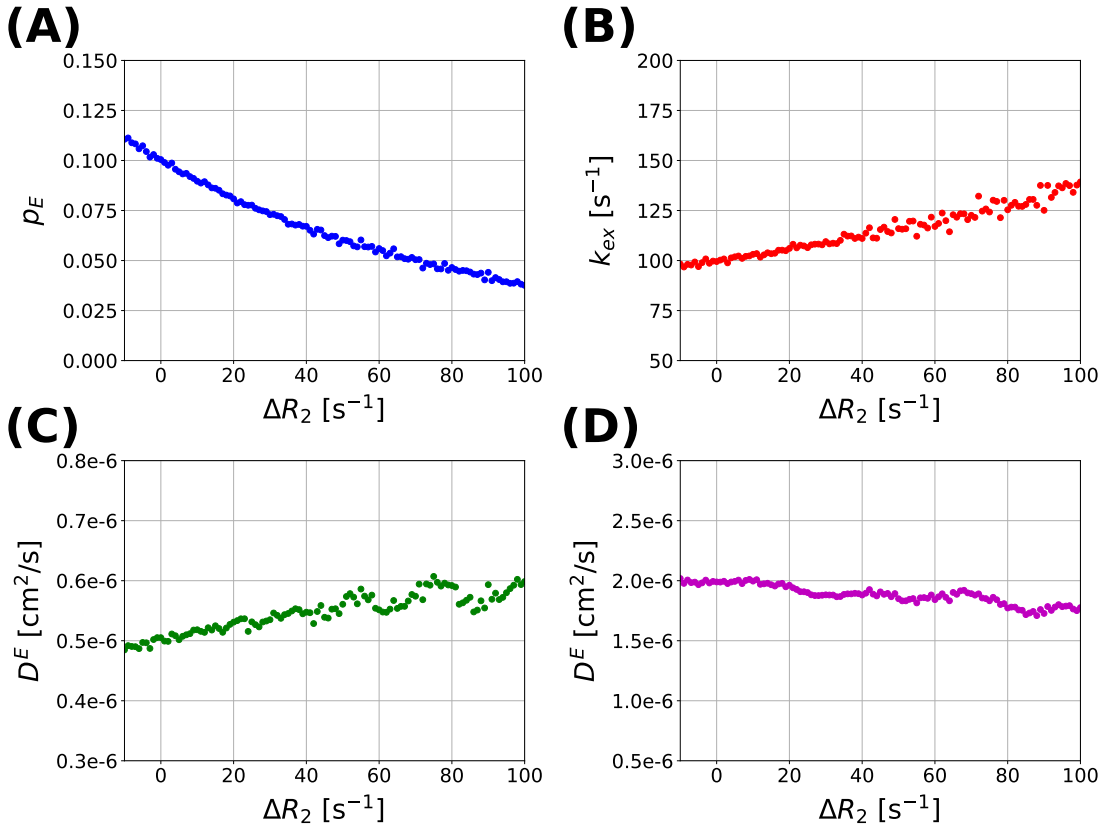

Figure S6. Errors in fitted exchange and excited state diffusion constant parameters caused by incorrect estimation of ΔR_2. Dispersion profiles were generated for a single residue at 14.0 T (no encode/decode gradients) and 18.8 T (without encode/decode gradients and with encode/decode gradients g = 50 G/cm, δ = 1 ms, see Figure 2), as in the experiment. The simulation parameters used were p_E = 0.10, k_{ex} = 100 s^{-1}, $\Delta\varpi_{GE}$ = 0.15 ppm, $R_1^G = R_1^E = 2$ s^{-1}, $R_2^G = R_2^E = 10$ s^{-1}, T_{relax} = 40 ms and $D^G = 1.0 \times 10^{-6}$ cm^2/s. The CPMG datasets were simulated with different ΔR_2 rates (x-axis) and fit with ΔR_2 = 0 s^{-1}. Panels (A) and (B) plot p_E and k_{ex} *vs* ΔR_2, respectively, with the corresponding D^E *vs* ΔR_2 profiles based on fitting simulated data with $D^E = 0.5 \times 10^{-6}$ cm^2/s (C) or 2.0×10^{-6} cm^2/s (D) plotted in Panels (C) and (D) respectively.

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```
/* CH3_1H_TQ_CPMG_diff_lek_800_cp

    This pulse sequence will allow one to perform the following experiment:

    2D 1H/13C 13CH3 TQ-CPMG to measure diffusion of the excited state

    Recorded as two experiments: with and without -Dref_flg. Without applies
     encode/decode gradients

    Assumes that sample is specifically 13CH3 labeled and 2H with 1H TQ CPMG applied

        1H: O1 on water (~4.7ppm) at end, most of the time at ~0.8 ppm (methyl 1H re
sonances)
            pwh = p1 1H pw90 @ power level pl1 highest power
            pwh_cp = p15 1H pw90 @ power level pl15 for CPMG pulses

       13C: O2 centre at 20 ppm
            pwc = p2 13C pw90 @ power level pl2 highest power
            power level pl21 is used for 13C decoupling.

        2H: O4 centre at ~0.8 ppm (methyl 1H resonances)
            pwd = p4 2H pw90 @ power level pl4 high power
            power level pl41 is used for 2H waltz-16 decoupling.
            Note: 2H decoupling only necessary for measuring nuCPMG < 100 Hz but is
used
                  throughout

    Written by T. Yuwen on Nov 21, 2016

    Modified by T. Yuwen on Nov 14, 2017 to add the option of ipap_flg: If ipap_flg
is defined and
      cal_flg undefined then it enters the CPMG period with equal amount of initial
IP and AP
      TQ magnetizations, therefore it is no longer necessary to correct for 13C R1 r
ates

    Modified by T. Yuwen on Nov 14, 2017 to change the phase phase cycle when comp18
0_flg is not defined:
      Always use xy-16 phase cycle instead of using mlev-4 sometimes

    Modified by T. Yuwen on Nov 16, 2017 to delete 'F3QF' when using cal_flg, in ord
er to simplify
      the setup for measuring differential relaxation between IP and AP TQ coherence
s, always keep the
      experiment as pseudo-3D but never goes to pseudo-4D

    Modified based on 13CH3_1H_TQ_CPMG_ty_600_cp, dephasing/rephasing gradients adde
d for diffusion
    measurement

      If cal_flg is used (recommend it not to be) then set ncyc_cp to > 40 and run 2
 expts with QF=2

    Recommend: use -Dcomp180_flg (to apply composite 180o CPMG pulses), -Dipap_flg (
runs CPMG
               with equal amounts of inphase and antiphase so that do not need to do
               correction for Cz), Dref_flg for 1 and not for the other CPMG series

*/

prosol relations=<triple>

#include <Avance.incl>
#include <Grad.incl>
#include <Delay.incl>

/***********************/
/*   Define phases     */
/***********************/
#define zero ph=0.0
#define one ph=90.0
#define two ph=180.0
#define three ph=270.0
```

```
/************************/
/*   Define pulses      */
/************************/
define pulse dly_pg1      /* Messerle purge pulse */
  "dly_pg1=5m"
define pulse dly_pg2      /* Messerle purge pulse */
  "dly_pg2=dly_pg1/1.62"
define pulse pwh
  "pwh=p1"                /* 1H hard pulse at power level p1 (tpwr) */
define pulse pwc
  "pwc=p2"                /* 13C pulse at power level pl2 (dhpwr) */

#ifdef Ddec
  define pulse pwd
    "pwd=p4"              /* 2H pulse at power pl4 */
#endif

define pulse pwh_cp       /* 1H CPMG pulse power level */
  "pwh_cp=p15"

#ifdef water_flg
  define pulse pw_sl1
    "pw_sl1=p14"          /* Eburp1 pulse, ~7000 us */
#endif

/************************/
/*   Define delays      */
/************************/
define delay hscuba       /* length of 1/2 scuba delay */
  "hscuba=30m"
define delay taua
  "taua=d3"               /* d3 ~ 1.8-2ms ~ 1.0s/(4*125.3)"  ~ 1 / 4J(CH) */
define delay taub
  "taub=d4"               /* d4 = 1/4JCH exactly */

define delay tauc
  "tauc=0.66m"

define delay time_T2
  "time_T2=d6"            /* CPMG duration <= 40 ms  */

"in0=inf1/2"
"d11=30m"
"d19=abs(1s/cnst1)/4"     /* Delay time for 3-9-19 WATERGATE */
"TAU2=0.2u"

/************************/
/*   Define f1180       */
/************************/
#ifdef f1180
  "d0=in0/2"
#else
  "d0=0.2u"
#endif

/************************************/
/* Define parameters related to CPMG */
/************************************/
define delay tauCPMG
define delay tauCPMG1
define delay tauCPMG2

#ifndef cal_flg
  define list<loopcounter> ncyc_cp=<$VCLIST>
#endif

/*****************************************************/
/* Assign cnsts to check validity of parameter ranges */
/*****************************************************/
#ifdef fsat
  "cnst10=plw10"          /* tsatpwr pl10 - set max at 0.00005W */
#endif
```

```
#ifdef mess_flg
  "cnst11=plw11"          /* tpwrmess pl11 - set max at 1.0W */
#endif

#ifdef water_flg
  "cnst14=spw14"          /* power level for eburp1 pulse preeceding start of sequenc
e */
#endif

"cnst15=plw15"            /* tpwrcp - power level for 1H CPMG pulses */
"cnst21=plw21"            /* dpwr pl21 - set max at 2.0W */

#ifdef Ddec
  "cnst4=plw4"            /* dpwr3 pl4 - set max at 10.5W */
  "cnst41=plw41"          /* dpwr3D pl41 - set max at 1.5W */
#endif

/************************/
/* Initialize variables */
/************************/
"l1=0"
"l2=0"
"spoal14=1"
"spoff14=0"

aqseq 321

#ifdef wgate_flg
  "acqt0=0"               /* select 'DIGIMOD = baseopt' to execute */
#else
  "acqt0=-pwh*2.0/PI"     /* select 'DIGIMOD = baseopt' to execute */
#endif

1 ze
/*********************************************************************/
/* Check validity of parameters and assign values to some of them */
/*********************************************************************/
#ifdef fsat
  if "cnst10 > 0.00005" {
    2u
    print "error: tpwrmess pl10 too large; < 0.00005W !!!"
    goto HaltAcqu
  }
#endif

#ifdef mess_flg
  if "cnst11 > 1" {
    2u
    print "error: tpwrmess pl11 too large; < 1W !!!"
    goto HaltAcqu
  }
#endif

#ifdef water_flg
  if "cnst14 > 0.01" {
    2u
    print "error: power level for eburp1 pulse is too large; < 0.01W !!!"
    goto HaltAcqu
  }
#endif

  if "cnst15 > 12" {
    2u
    print "error: 1H CPMG power pl15 too large; < 12W !!!"
    goto HaltAcqu
  }

  if "time_T2 > 40.1m" {
    2u
    print "error: time_T2 too long; < 41ms !!!"
    goto HaltAcqu
  }
```

```
  if "cnst21 > 1.5" {
    2u
    print "error: dpwr pl21 too large; < 1.5W !!!"
    goto HaltAcqu
  }

  if "aq > 64m" {
    2u
    print "error: aq is too long; < 64ms !!!"
    goto HaltAcqu
  }

#ifdef Ddec
  if "cnst4 > 15" {
    2u
    print "error: dpwr3 pl4 too large; < 15W !!!"
    goto HaltAcqu
  }

  if "cnst41 > 4" {
    2u
    print "error: dpwr3D pl41 too large; < 4W !!!"
    goto HaltAcqu
  }

  if "pwd > 160u" {
    2u
    print "error: pwd is too large; < 160us !!!"
    goto HaltAcqu
  }

  if "pwd < 100u" {
    2u
    print "error: pwd is too small; > 100us !!!"
    goto HaltAcqu
  }

  if "d1 < 2.0s" {
    2u
    print "error: d1 is too short when applying 2H decoupling; > 2s !!!"
    goto HaltAcqu
  }

; d11 LOCKDEC_ON /* Not required for AvanceIII-HD */
  50u LOCKH_ON
  d11 H2_PULSE
  4u pl41:f4
#endif

2 d11 do:f2
/*************************/
/* Update list pointers */
/*************************/
  2u
#ifndef cal_flg
  "ncyc_cp.idx=l1"
#endif
  2u rpp11 rpp12 rpp13 rpp14

/***************************************/
/* Continue to check run time variables */
/***************************************/
#ifdef cal_flg
  if "l2 < 1" {
    2u
    print "error: ncyc_cp should be set to positive value when using cal_flg !!!"
    goto HaltAcqu
  }

  if "time_T2/l2 > 1.01m" {
    2u
    print "error: nu_cpmg should be set to >1000 Hz when using cal_flg !!!"
```

```
    goto HaltAcqu
  }
#else
  "l2 = (trunc(ncyc_cp + 0.3))"
#endif

  if "l2 > 80" {
    2u
    print "error: ncyc_cp must be < 81 !!!"
    goto HaltAcqu
  }

  if "l2 != 0" {
    "tauCPMG = time_T2*0.25/l2"
    if "tauCPMG < 124.0u" {
      2u
      print "error: tauCPMG < 124u too short for CPMG !!!"
      goto HaltAcqu
    }
  }
  else {
    "tauCPMG = time_T2*0.25"
  }

  "tauCPMG1 = tauCPMG - pwh_cp*2.0"
#ifdef comp180_flg
  "tauCPMG2 = tauCPMG - pwh_cp*2.0"
#else
  "tauCPMG2 = tauCPMG - pwh_cp"
#endif

/******************/
/* Messerle purge */
/******************/
#ifdef mess_flg
  4u pl11:f1
  (dly_pg1 ph26):f1
  2u
  (dly_pg2 ph27):f1
#endif

/*****************/
/* Presaturation */
/*****************/
#ifdef Ddec
  "DELTA = d1 - 300m - d11 - 6m"

#ifdef fsat
  4u pl10:f1
  300m cw:f1 ph26
  2u do:f1
  d11 H2_LOCK
  6m LOCKH_OFF
  DELTA cw:f1 ph26
  2u do:f1
  4u pl1:f1
#ifdef fscuba
  hscuba
  (pwh ph26):f1
  (pwh*2 ph27):f1
  (pwh ph26):f1
  hscuba
#endif /*fscuba*/
#else  /*fsat*/
  4u pl1:f1
  300m
  d11 H2_LOCK
  6m LOCKH_OFF
  DELTA
#endif /*fsat*/
  50u LOCKH_ON
  15u H2_PULSE
  20u UNBLKGRAMP
```

```
#else /*Ddec*/
#ifdef fsat
  4u pl10:f1
  d1 cw:f1 ph26
  2u do:f1
  4u pl1:f1
#ifdef fscuba
  hscuba
  (pwh ph26):f1
  (pwh*2 ph27):f1
  (pwh ph26):f1
  hscuba
#endif /*fscuba*/
#else  /*fsat*/
  4u pl1:f1
  d1
#endif /*fsat*/
  20u UNBLKGRAD
#endif /*Ddec*/

/**************************/
/* Water selective Eburp1 */
/**************************/
#ifdef water_flg
  2u
  (pw_sl1:sp14 ph26):f1

  2u
  p50:gp0
  d16
#endif

/***************************************/
/* Destroy 13C equlibrium magnetization */
/***************************************/
  4u pl2:f2
  (pwc ph26):f2

  2u
  p50:gp0
  d16

/************************/
/* Create TQ coherence */
/************************/
  10u fq=cnst1:f1
  4u pl15:f1

  (pwh_cp ph1):f1

  2u
  p51:gp1
  d16

  "DELTA = taua - 2u - p51 - d16 - pwh_cp*2.0/PI"
  DELTA

  (center (pwh_cp*2 ph1):f1 (pwc*2 ph26):f2)

  2u
  p51:gp1
  d16

  "DELTA = taua - 2u - p51 - d16"
  DELTA

  (pwc ph26):f2

  2u
  p52:gp2
  d16
```

```
  "DELTA = taub - 2u - p52 - d16"
  DELTA

  (center (pwh_cp*2 ph1):f1 (pwc*2 ph26):f2)

  2u
  p52:gp2
  d16

  "DELTA = taub - 2u - p52 - d16"
  DELTA

  (pwc ph26):f2
  (pwh_cp ph1):f1

/*************************************************************/
/* Option to measure differential relaxation for AP/IP TQ */
/*************************************************************/
#ifdef cal_flg
  if "l1%2 == 0" {
    "DELTA = tauc - pwh_cp*2.0"
    DELTA
    (pwh_cp ph29 pwh_cp*2 ph26 pwh_cp ph29):f1
    DELTA
  }
  else {
    tauc
    tauc
  }
#else
#ifdef ipap_flg
  tauc
#endif /*ipap_flg*/
#endif /*cal_flg*/

/*************************************************************/
/* Additional encode gradients for diffusion measurement */
/*************************************************************/
#ifdef Ddec
  "DELTA = 4u + pwd + 2u + 2u"
  DELTA
#endif

#ifdef ref_flg
  "DELTA = 2u + p58 + d17"
  DELTA
#else
  2u
  p58:gp8
  d17
#endif

  (pwh_cp ph29 pwh_cp*2 ph26 pwh_cp ph29):f1

#ifdef ref_flg
  "DELTA = 2u + p58 + d17"
  DELTA
#else
  2u
  p58:gp8*-1
  d17
#endif

#ifdef Ddec
  4u pl4:f4
  (pwd ph27):f4
  2u pl41:f4
  (2u cpds4 ph26):f4
#endif

/*********************************/
/* The first half of CPMG period */
/*********************************/
```

```
  if "l2 == 1" {
    tauCPMG1
    (pwh_cp ph29 pwh_cp*2 ph26 pwh_cp ph29):f1
    tauCPMG1
  }

  if "l2 > 1" {
3   tauCPMG2
#ifdef comp180_flg
    (pwh_cp ph14 pwh_cp*2 ph11 pwh_cp ph14):f1
#else
    (pwh_cp*2 ph11):f1
#endif
    tauCPMG2 ipp11 ipp12 ipp13 ipp14
    lo to 3 times l2
  }

/*****************************/
/* The central 180o 1H pulse */
/*****************************/
  (pwh_cp*2 ph2):f1

/***********************************/
/* The second half of CPMG period */
/***********************************/
  if "l2 == 1" {
    tauCPMG1
    (pwh_cp ph27 pwh_cp*2 ph26 pwh_cp ph27):f1
    tauCPMG1
  }

  if "l2 > 1" {
4   tauCPMG2 dpp11 dpp12 dpp13 dpp14
#ifdef comp180_flg
    (pwh_cp ph13 pwh_cp*2 ph12 pwh_cp ph13):f1
#else
    (pwh_cp*2 ph12):f1
#endif
    tauCPMG2
    lo to 4 times l2
  }

/*************************************************************/
/* Additional decode gradients for diffusion measurement */
/*************************************************************/
#ifdef Ddec
  2u do:f4
  2u pl4:f4
  (pwd ph29):f4
#endif

#ifdef ref_flg
  "DELTA = 2u + p58 + d17"
  DELTA
#else
  2u
  p58:gp8*-1
  d17
#endif

  (pwh_cp ph27 pwh_cp*2 ph26 pwh_cp ph27):f1

#ifdef ref_flg
  "DELTA = 2u + p58 + d17"
  DELTA
#else
  2u
  p58:gp8
  d17
#endif

#ifdef Ddec
  "DELTA = 2u + 2u + pwd"
```

```
  DELTA
#endif

/*************************************************************/
/* Option to measure differential relaxation for AP/IP TQ */
/*************************************************************/
#ifdef cal_flg
  if "l1%2 == 0" {
    "DELTA = tauc - pwh_cp*2.0"
    DELTA
    (pwh_cp ph27 pwh_cp*2 ph26 pwh_cp ph27):f1
    DELTA
  }
  else {
    tauc
    tauc
  }
#else
#ifdef ipap_flg
  tauc
#endif /*ipap_flg*/
#endif /*cal_flg*/

  (pwh_cp ph26):f1
  (pwc ph3):f2

  2u
  p53:gp3
  d16

  "DELTA = taub - 2u - p53 - d16"
  DELTA

/*********************************/
/* Option to record HMQC or HSQC */
/*********************************/
#ifdef mq_flg
  (pwc*2 ph26):f2

  "TAU1=larger(d0-pwh_cp*2.0,TAU2)"
  TAU1

  (pwh_cp ph29 pwh_cp*2 ph26 pwh_cp ph29):f1

  TAU1

  2u
  p53:gp3
  d16

  "DELTA = taub - 2u - p53 - d16"
  DELTA

  (pwc ph4):f2

  "DELTA = pwc*2.0"
  DELTA

  (pwh_cp ph27):f1

#else
  (center (pwh_cp ph29 pwh_cp*2 ph26 pwh_cp ph29):f1 (pwc*2 ph26):f2)

  2u
  p53:gp3
  d16

  "DELTA = taub - 2u - p53 - d16"
  DELTA

  (pwc ph26):f2
  (pwh_cp ph27):f1
```

```
  2u
  p56:gp6
  d16

  (pwc ph26):f2

  "TAU1=larger(d0-pwh_cp*2.0-pwc*2.0/PI,TAU2)"
  TAU1

  (pwh_cp ph29 pwh_cp*2 ph26 pwh_cp ph29):f1

  TAU1

  (pwc ph4):f2
#endif

  2u
  p54:gp4
  d16

/*****************************************************************/
/* C->H back transfer, use wgate_flg for better water suppression */
/*****************************************************************/
  10u fq=0:f1
  4u pl1:f1

  (pwh ph26):f1

#ifdef wgate_flg
  2u
  p57:gp7
  d16

  "DELTA = taua - 2u - p57 - d16 - d19*3.367 - pwh*2.0/PI"
  DELTA

  (center
  (pwh*0.231 ph26 d19*2 pwh*0.692 ph26 d19*2 pwh*1.462 ph26 d19*2 pwh*1.462 ph28 d19
*2 pwh*0.692 ph28 d19*2 pwh*0.231 ph28):f1
  (pwc*2 ph26):f2)

  2u
  p57:gp7
  d16

  "DELTA = taua - 2u - p57 - d16 - d19*3.367 - 4u - pwc*2.0 - 4u"
  DELTA
#else
  2u
  p55:gp5
  d16

  "DELTA = taua - 2u - p55 - d16"
  DELTA

  (center (pwh*2 ph26):f1 (pwc*2 ph26):f2)

  2u
  p55:gp5
  d16

  "DELTA = taua - 2u - p55 - d16 - 4u - pwc*2.0 - 4u"
  DELTA
#endif

#ifdef Ddec
  4u BLKGRAMP
#else
  4u BLKGRAD
#endif

  (pwc ph26):f2
  (pwc ph5):f2
```

```
  4u pl21:f2             /* lower power for 13C decoupling */

#ifndef wgate_flg
  (pwh ph26):f1
#endif

/*******************************/
/* Signal detection and looping */
/*******************************/
  go=2 ph31 cpds2:f2
  d11 do:f2 mc #0 to 2

  F2QF(calclc(l1, 1))
  F1PH(calph(ph4, +90), caldel(d0, +in0) & calph(ph4, +180) & calph(ph31, +180))

#ifdef Ddec
  d11 H2_LOCK
  d11 LOCKH_OFF
; d11 LOCKDEC_OFF        /* use statement for earlier hardware */
#endif

HaltAcqu, 1m
exit

ph0=1
ph1=(6) 0 1 2 3 4 5
ph2=1 3
ph3={{0}*6}^2
ph4={{0}*12}^2
ph5=0 2
ph11={0 1 0 1 1 0 1 0}^2
ph12={0 3 0 3 3 0 3 0}^2
ph13=ph12+ph0
ph14=ph11-ph0
ph26=0
ph27=1
ph28=2
ph29=3
ph31={{{0 2}*3}^2}^2

;d1: Repetition delay D1
;d3: taua ~1/(4*JCH)  ~1.8-2.0ms
;d4: taub - set to 1/4JHC = 2.0 ms
;d6: time_T2 CPMG duration <= 40ms
;d11: delay for disk i/o, 30ms
;d16: gradient recovery delay, 200us
;d17: gradient recovery delay, 500us
;d19: delay for binomial water suppression, = 1/(4*|cnst1|)
;pl1: tpwr - power level for pwh
;pl2: dhpwr - power level for 13C pulse pwc (p2)
;pl4: power level for 2H high power pulses
;pl10: tsatpwr - power level for presat
;pl11: tpwrmess - power level for Messerle purge
;pl15: power level for 1H CPMG pulses pwh_cp
;pl21: dpwr - power level for  13C decoupling cpd2
;pl41: power level for 2H waltz decoupling
;sp14: power level for eburp1 pulse
;spnam14: eburp1 pulse on water
;p1: pwh
;p2: pwc
;p4: 2H high power pulse
;p14: eburp1 pulse width, typically 7000us
;p15: 1H pw for CPMG pulses
;p50: gradient pulse 50                                  [1000 usec]
;p51: gradient pulse 51                                  [400 usec]
;p52: gradient pulse 52                                  [600 usec]
;p53: gradient pulse 53                                  [300 usec]
;p54: gradient pulse 54                                  [500 usec]
;p55: gradient pulse 55                                  [300 usec]
;p56: gradient pulse 56                                  [500 usec]
;p57: gradient pulse 57                                  [800 usec]
;p58: gradient pulse for diffusion measure
```

```
;cpdprg2: 13C decoupling program during t2 [waltz16]
;pcpd2: 13C pulse width for 13C decoupling
;cpdprg4: 2H decoupling program during t1 [waltz16]
;pcpd4: 2H pulse width for 2H decoupling
;cnst1: offset (Hz) of methyls from water
;vclist: variable counter list for ncyc_cp
;inf1: 1/SW(X) = 2*DW(X)
;in0: 1/(2*SW(x))=DW(X)
;nd0: 2
;ns: 6*n
;FnMODE: States in F1
;FnMODE: QF in F2

;for z-only gradients:
;gpz0: 20%
;gpz1: 25%
;gpz2: 30%
;gpz3: -25%
;gpz4: 50%
;gpz5: -40%
;gpz6: -75%
;gpz7: -80%
;gpz8: gradient to be used for diffusion measure

;use gradient files:
;gpnam0: SMSQ10.32
;gpnam1: SMSQ10.32
;gpnam2: SMSQ10.32
;gpnam3: SMSQ10.32
;gpnam4: SMSQ10.32
;gpnam5: SMSQ10.32
;gpnam6: SMSQ10.32
;gpnam7: SMSQ10.32
;gpnam8: SMSQ10.32

;zgoptns: Dfsat, Dmess_flg, Dfscuba, Dwater_flg, Dwgate_flg, Df1180, DDdec, Dcomp180
_flg, Dmq_flg, Dcal_flg, Dipap_flg, Dref_flg
```