Supporting information for
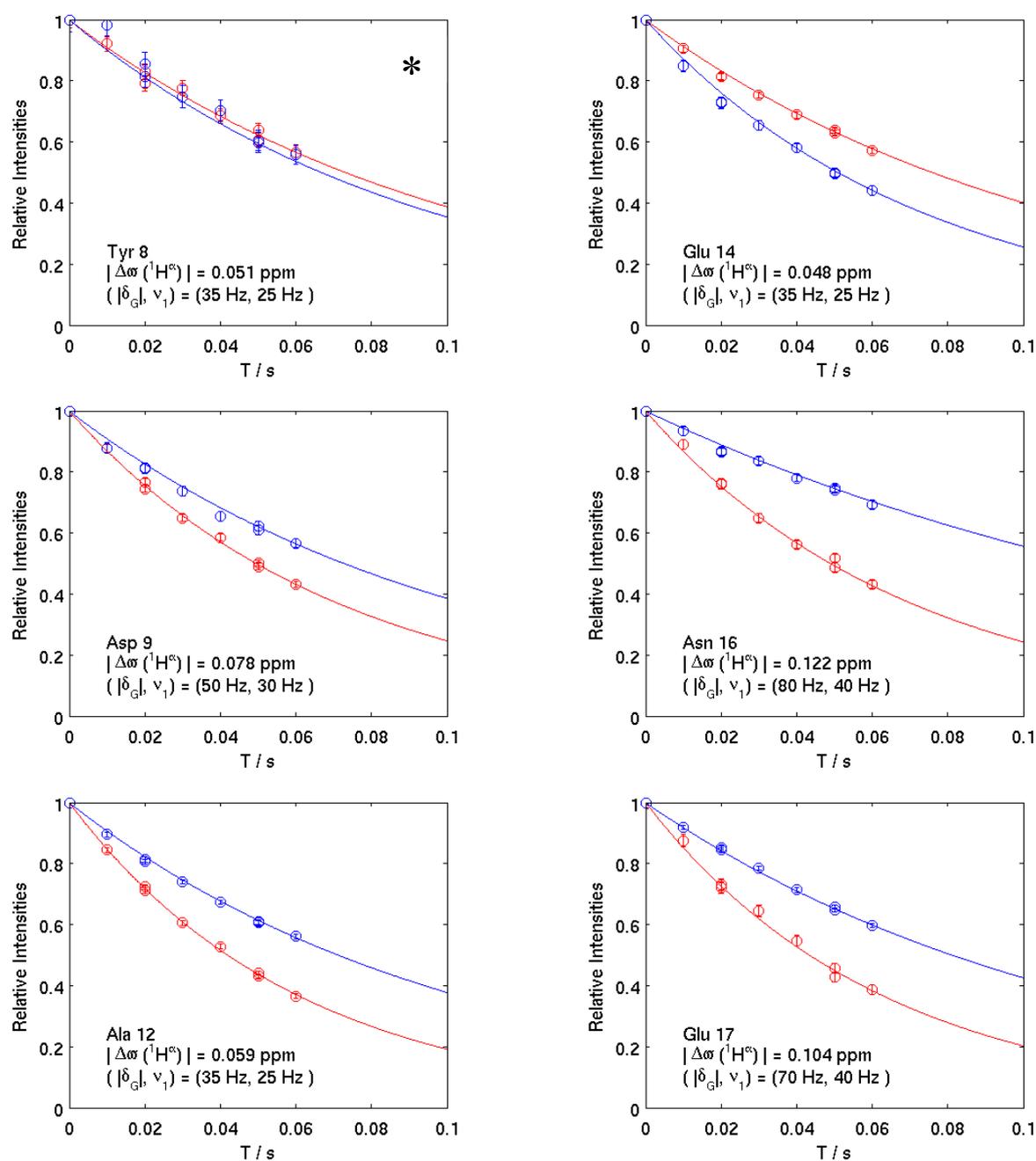
Measuring the Signs of ^{1}H$^\alpha$ Chemical Shift Differences Between Ground and Excited Protein States by Off-Resonance Spin-Lock R$_{1\rho}$ NMR Spectroscopy

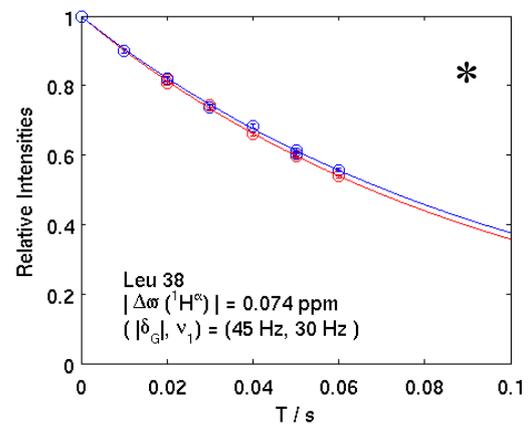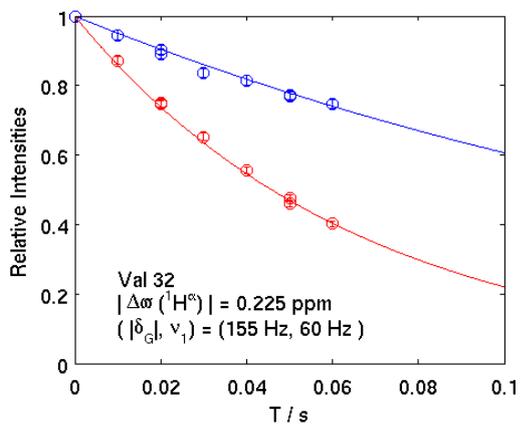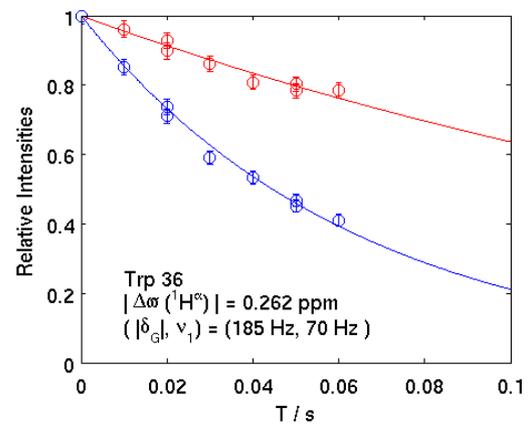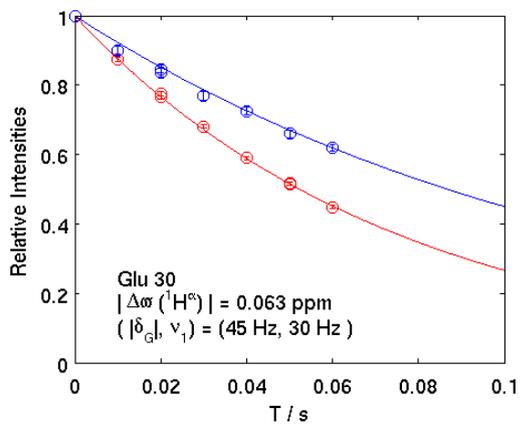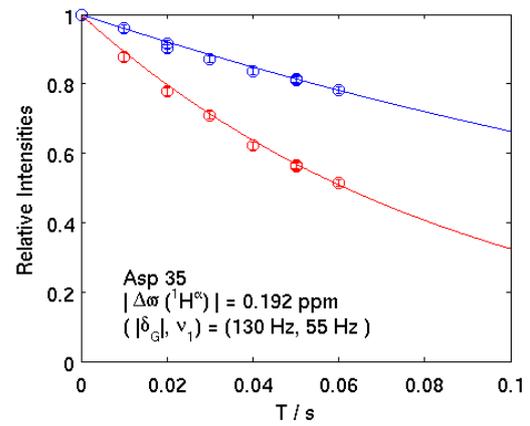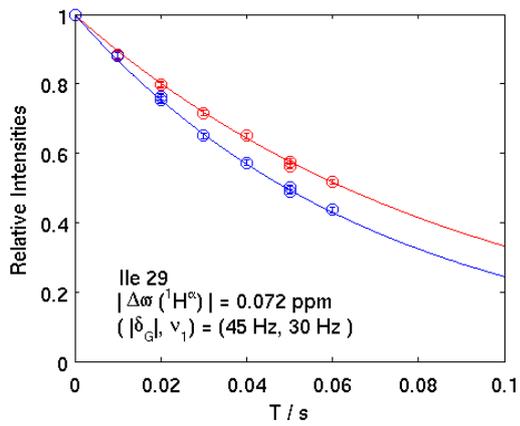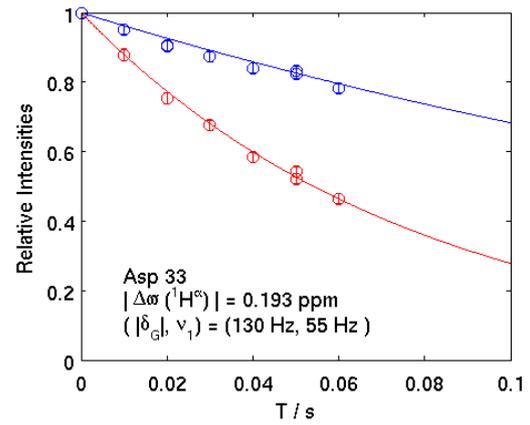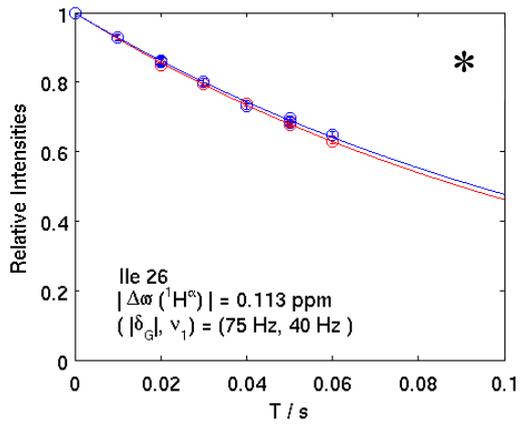Renate Auer [1,2], Philipp Neudecker[2], D. Ranjith Muhandiram[2], Patrik Lundström[2,3], D. Flemming Hansen[2], Robert Konrat[1] and Lewis E. Kay[2]
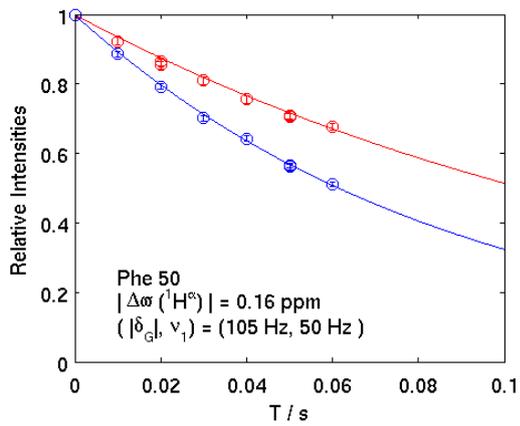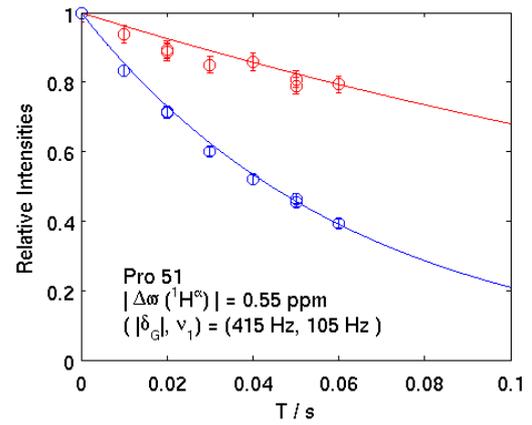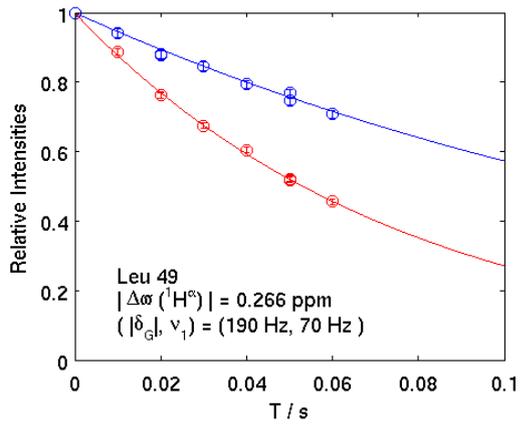
[1]*Department of Structural and Computational Biology, Max F. Perutz Laboratories, University of Vienna, Campus-Vienna-Biocenter 5, A-1030 Vienna, Austria,* [2]*Departments of Molecular Genetics, Biochemistry and Chemistry, University of Toronto, Toronto, Ontario, Canada, M5S 1A8 and* [3]*Molecular Biotechnology/IFM Linköping University, SE-581 83 Linköping, Sweden*

Details of Pulse Scheme (Figure 1): ^{1}H and ^{13}C carrier frequencies are placed initially on resonance for the peak of interest; subsequently the ^{1}H carrier is jumped to the position of the spin-lock field immediately prior to the pulse of phase ϕ4. All solid pulses have flip angles of 90° and are applied along the x-axis, unless indicated otherwise. Simultaneous ^{1}H/^{13}C spin-lock fields (140 Hz) are applied for durations of 1/J$_{CH}$ (between points *a,b* and *c,d*) where J$_{CH}$ is the one-bond ^{1}H$^\alpha$-^{13}C$^\alpha$ scalar coupling constant ($\approx$ 140 Hz). Immediately after gradient 1 ^{1}H purge pulses are applied (17 kHz) for durations of 2 ms (x-axis) and 1 ms (y-axis) to eliminate residual water signal. ^{1}H pulses of phase ϕ4/ϕ5 are applied with a flip angle θ such that tanθ = ω_I/|δ_G|, using (δ_G,ω_I) optimized as described in the text. During the spin-lock period an on-resonance ^{13}C continuous-wave decoupling field of 2.5 kHz is applied to eliminate scalar coupling modulations and cross-correlation effects between ^{1}H-^{13}C dipolar and ^{1}H CSA interactions. The delay τ_{eq} is set to 5 ms to ensure that the magnetization from each of the exchanging states corresponds faithfully to the equilibrium distribution[1]. ^{13}C decoupling during acquisition is achieved with a WALTZ-16 field[2]. The phase cycle is: ϕ_1=(y,-y), ϕ_2=2(x),2(-x), ϕ_3=4(x),4(-x), ϕ_6 = x,2(-x),x-x,2(x),-x. For the spin-lock carrier upfield(downfield) of the ground state resonance (on Varian spectrometers) ϕ_4=y(-y) and ϕ_5= -y(y). Gradient strengths and durations are (ms,G/cm): 0=(1,7.5), 1=(0.5,10), 2=(0.8,15), 3=(0.6,-4), 4=(0.2,-4), 5=(0.5,8).

Figure S1. $R_{1\rho}$ values measured using the pulse scheme of Figure 1 (800 MHz) for offsets downfield (red) and upfield (blue) of the major peak. The exchanging system is the Abp1p SH3 domain + 6% Ark1p peptide[3]. $^1H^\alpha$ $|\Delta\varpi|$ values obtained from analysis of CPMG relaxation dispersion experiments[4] are indicated in each panel, along with optimal $(|\delta_G|, \nu_1=\omega_1/2\pi)$ values, calculated as described in the text (for a spectrometer field of 800 MHz). The solid lines were obtained from a fit of the data to an exponential decay function, error bars were extracted from duplicate data points. Those panels indicated by * show results from residues whose $\Delta\omega$ values are overestimated from CPMG relaxation dispersion measurements (see Table T1). For these residues it is not possible to confidently predict the sign of $\Delta\varpi$ since $R_{1\rho}^{\pm}$ are not sufficiently different.

Ile 26
$| \Delta \varpi (^1H^\alpha) | = 0.113$ ppm
$(|\delta_G|, \nu_1) = (75$ Hz, 40 Hz $)$

Asp 33
$| \Delta \varpi (^1H^\alpha) | = 0.193$ ppm
$(|\delta_G|, \nu_1) = (130$ Hz, 55 Hz $)$

Ile 29
$| \Delta \varpi (^1H^\alpha) | = 0.072$ ppm
$(|\delta_G|, \nu_1) = (45$ Hz, 30 Hz $)$

Asp 35
$| \Delta \varpi (^1H^\alpha) | = 0.192$ ppm
$(|\delta_G|, \nu_1) = (130$ Hz, 55 Hz $)$

Glu 30
$| \Delta \varpi (^1H^\alpha) | = 0.063$ ppm
$(|\delta_G|, \nu_1) = (45$ Hz, 30 Hz $)$

Trp 36
$| \Delta \varpi (^1H^\alpha) | = 0.262$ ppm
$(|\delta_G|, \nu_1) = (185$ Hz, 70 Hz $)$

Val 32
$| \Delta \varpi (^1H^\alpha) | = 0.225$ ppm
$(|\delta_G|, \nu_1) = (155$ Hz, 60 Hz $)$

Leu 38
$| \Delta \varpi (^1H^\alpha) | = 0.074$ ppm
$(|\delta_G|, \nu_1) = (45$ Hz, 30 Hz $)$

S3

Leu 49
$|\Delta\varpi\ (^1H^\alpha)| = 0.266$ ppm
$(\ |\delta_G|, \nu_1\ ) = (190\ Hz,\ 70\ Hz\ )$

Pro 51
$|\Delta\varpi\ (^1H^\alpha)| = 0.55$ ppm
$(\ |\delta_G|, \nu_1\ ) = (415\ Hz,\ 105\ Hz\ )$

Phe 50
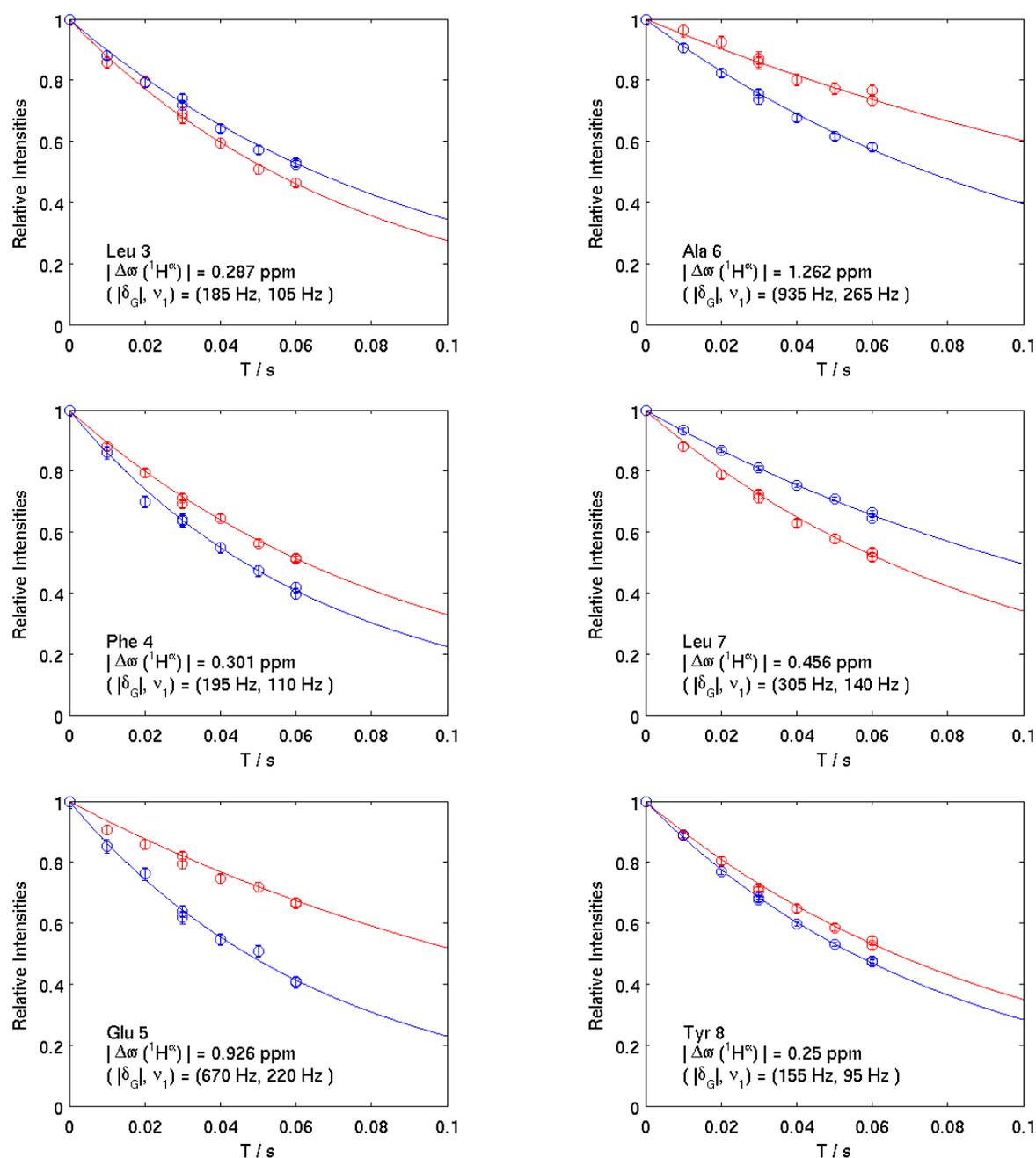$|\Delta\varpi\ (^1H^\alpha)| = 0.16$ ppm
$(\ |\delta_G|, \nu_1\ ) = (105\ Hz,\ 50\ Hz\ )$

Each data point was obtained in approximately 6 minutes (room temperature probe-head), providing excellent signal-to-noise (≈1 mM sample).

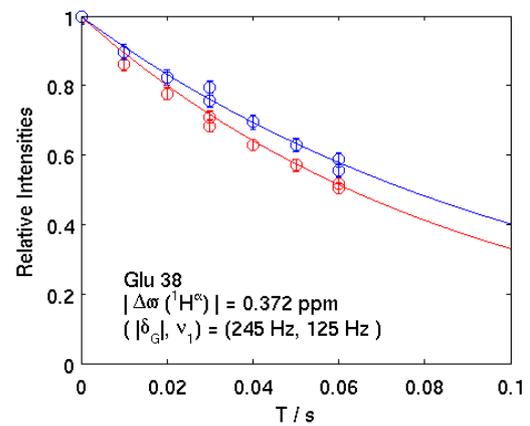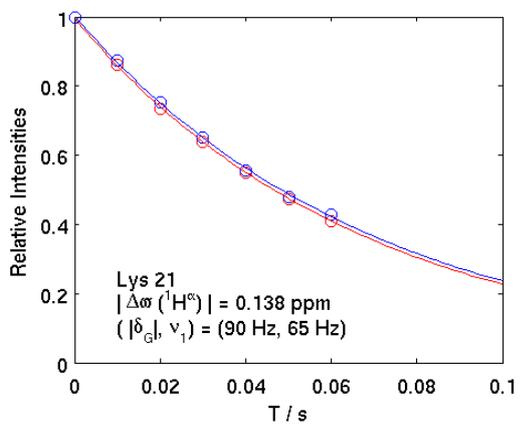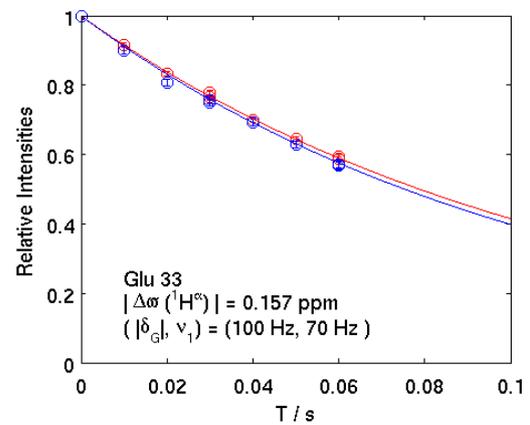**Table T1**: 17 residues in the Abp1p SH3 domain, 6% Ark1p sample for which $^1H^\alpha$ $\Delta\varpi$ values were measured via CPMG relaxation dispersion experiments[4]. First column shows signed $\Delta\varpi$ values (ppm) obtained directly from spectra of the ligand-free and bound Abp1p SH3 domains. Second column indicates $|\Delta\varpi|$ values from the $^1H^\alpha$ CPMG experiment[4] along with signs obtained from off-resonance $R_{1\rho}$ measurements using the pulse sequence of Figure 1. Sign information ('no sign') could not be obtained for residues 8, 26 and 38 ('no sign'); these residues have the smallest $|\Delta\varpi|$ values of the residues considered (see first column, $\Delta\varpi$ (Direct)) and the lowest t-test values (last column).

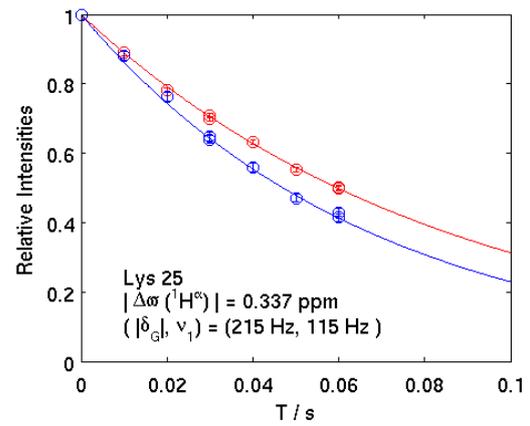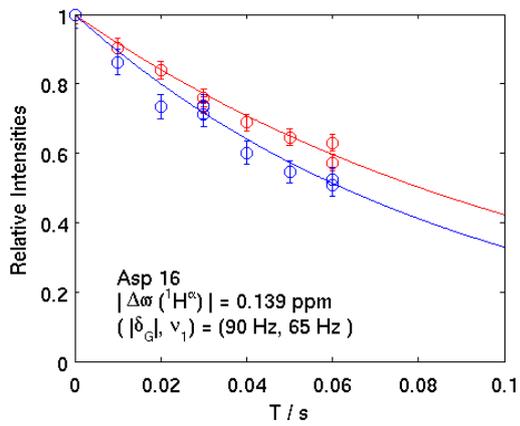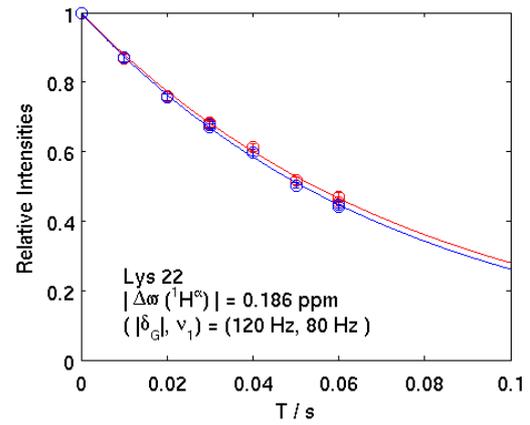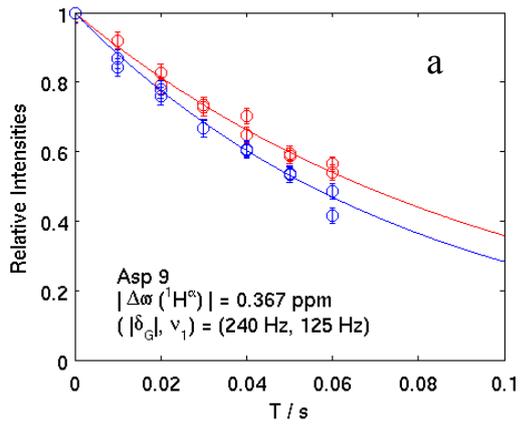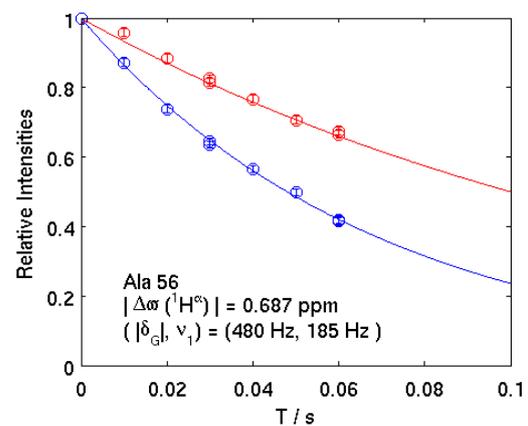| Residue	$\Delta\varpi$ (Direct) / ppm	$\Delta\varpi$ (Extracted) / ppm	t value[a]
Val 32	+ 0.218	+ 0.225 ± 0.005	32
Leu 49	+ 0.211	+ 0.266 ± 0.006	29
Asp 35	+ 0.199	+ 0.192 ± 0.005	26
Ala 12	+ 0.059	+ 0.059 ± 0.005	26
Glu 30	+ 0.055	+ 0.063 ± 0.005	24
Pro 51	- 0.541	- 0.55 ± 0.01	23
Asp 33	+ 0.188	+ 0.193 ± 0.005	22
Asn 16	+ 0.116	+ 0.122 ± 0.004	21
Phe 50	- 0.132	- 0.160 ± 0.006	20
Trp 36	- 0.293	- 0.262 ± 0.006	17
Glu 17	+ 0.083	+ 0.104 ± 0.004	15
Glu 14	- 0.063	- 0.048 ± 0.007	12
Asp 9	+ 0.061	+ 0.078 ± 0.007	12
Ile 29	- 0.050	- 0.072 ± 0.004	12
Leu 38	- 0.012	0.074 ± 0.004 (no sign)	3
Ile 26	- 0.028	0.113 ± 0.004 (no sign)	2
Tyr 8	- 0.031	0.051 ± 0.005 (no sign)	1

[a] Student's t-test value to evaluate whether $R_{1\rho}^{\pm}$ differ based on equations (18.1) and (18.2) of reference 5. Values $t > t_{\alpha(2),n}$ indicate a statistically significant difference at a confidence limit of $> (1-\alpha)$ with n the number of degrees of freedom n=2N-4 (N is the number of points measured per decay curve). For $t \approx t_{\alpha(2),n}$ (shaded) it is important to inspect the decay profiles closely prior to choosing the sign. For $t < t_{\alpha(2),n}$ decay curves for $R_{1\rho}^{\pm}$ are essentially superimposed and no sign information is available. We chose $\alpha=0.05$ corresponding to a confidence level of 95% which gives $t_{0.05(2),14} = 2.15$.

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Figure S2. $R_{1\rho}$ values measured using the pulse scheme of Figure 1 for offsets downfield (red) and upfield (blue) of the major peak. The exchanging system is the A39V/N53P/V55L Fyn SH3 domain[5]. $^1H^\alpha$ $|\Delta\varpi|$ values obtained from analysis of CPMG relaxation dispersion experiments are indicated in each panel, along with optimal ($|\delta_G|$,$\nu_1=\omega_1/2\pi$) values, calculated as described in the text (for a spectrometer field of 800 MHz). The solid lines were obtained from a fit of the data to an exponential decay function, error bars were extracted from duplicate data points.

a

Asp 9
$| \Delta \varpi \, (^1H^\alpha) | = 0.367$ ppm
$(| \delta_G |, \nu_1) = (240$ Hz, 125 Hz$)$

Lys 22
$| \Delta \varpi \, (^1H^\alpha) | = 0.186$ ppm
$(| \delta_G |, \nu_1) = (120$ Hz, 80 Hz $)$

Asp 16
$| \Delta \varpi \, (^1H^\alpha) | = 0.139$ ppm
$(| \delta_G |, \nu_1) = (90$ Hz, 65 Hz $)$

Lys 25
$| \Delta \varpi \, (^1H^\alpha) | = 0.337$ ppm
$(| \delta_G |, \nu_1) = (215$ Hz, 115 Hz $)$

Leu 18
$| \Delta \varpi \, (^1H^\alpha) | = 0.135$ ppm
$(| \delta_G |, \nu_1) = (90$ Hz, 65 Hz $)$

Glu 33
$| \Delta \varpi \, (^1H^\alpha) | = 0.157$ ppm
$(| \delta_G |, \nu_1) = (100$ Hz, 70 Hz $)$

Lys 21
$| \Delta \varpi \, (^1H^\alpha) | = 0.138$ ppm
$(| \delta_G |, \nu_1) = (90$ Hz, 65 Hz$)$

Glu 38
$| \Delta \varpi \, (^1H^\alpha) | = 0.372$ ppm
$(| \delta_G |, \nu_1) = (245$ Hz, 125 Hz $)$

Asp 40
$| \Delta \varpi (^1H^\alpha) | = 0.244$ ppm
$(|\delta_G|, \nu_1) = (155$ Hz, 95 Hz$)$

Ser 52
$| \Delta \varpi (^{15}N) | = 0.217$ ppm
$(|\delta_G|, \nu_1) = (70$ Hz, 150 Hz $)$

Thr 47
$| \Delta \varpi (^1H^\alpha) | = 0.166$ ppm
$(|\delta_G|, \nu_1) = (105$ Hz, 75 Hz $)$

Pro 53
$| \Delta \varpi (^{15}N) | = 0.296$ ppm
$(|\delta_G|, \nu_1) = (90$ Hz, 210 Hz $)$

Tyr 49
$| \Delta \varpi (^1H^\alpha) | = 0.342$ ppm
$(|\delta_G|, \nu_1) = (220$ Hz, 115 Hz $)$

Tyr 54
$| \Delta \varpi (^1H^\alpha) | = 0.133$ ppm
$(|\delta_G|, \nu_1) = (90$ Hz, 65 Hz $)$

Ile 50
$| \Delta \varpi (^1H^\alpha) | = 0.404$ ppm
$(|\delta_G|, \nu_1) = (265$ Hz, 130 Hz $)$

Ala 56
$| \Delta \varpi (^1H^\alpha) | = 0.687$ ppm
$(|\delta_G|, \nu_1) = (480$ Hz, 185 Hz $)$

Pro 57
$| \Delta \varpi \, (^{1}H^{\alpha}) | = 0.813$ ppm
$(|\delta_{G}|, \nu_{1}) = (580$ Hz$, 205$ Hz $)$

Val 58
$| \Delta \varpi \, (^{1}H^{\alpha}) | = 0.254$ ppm
$(|\delta_{G}|, \nu_{1}) = (160$ Hz$, 95$ Hz $)$
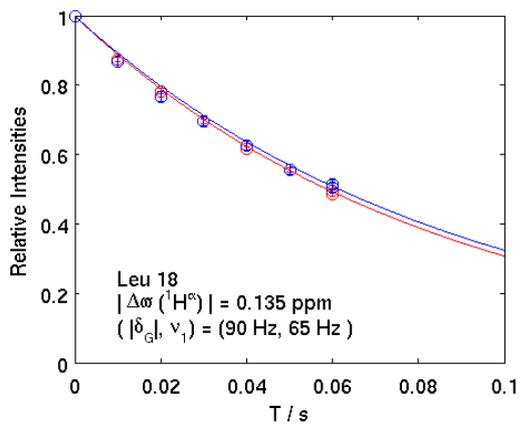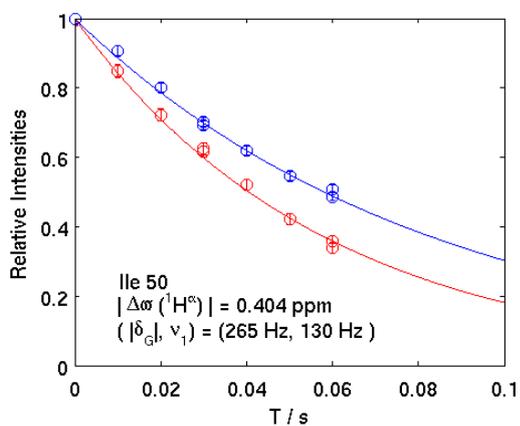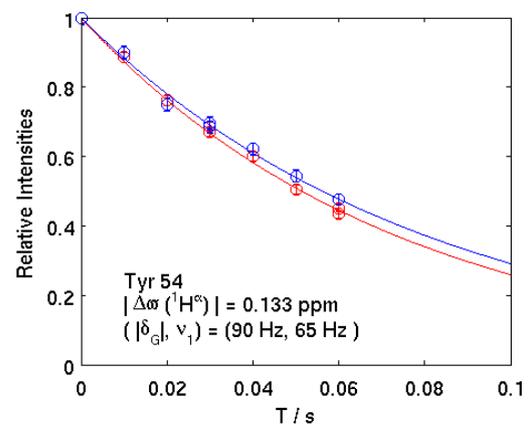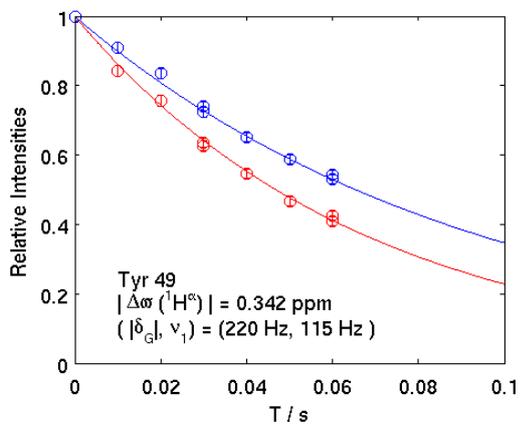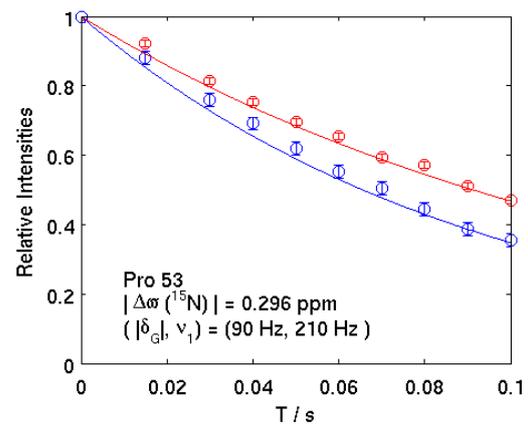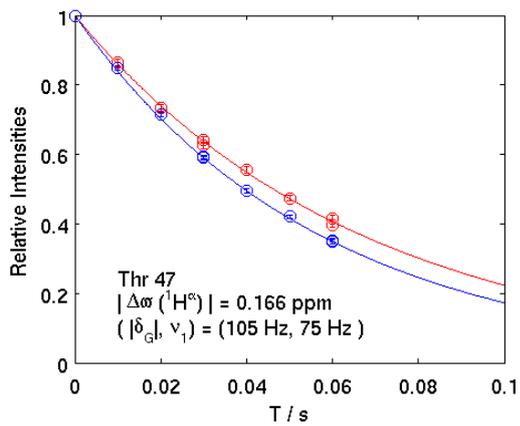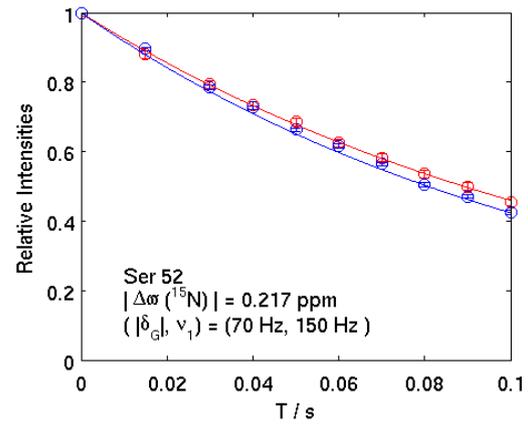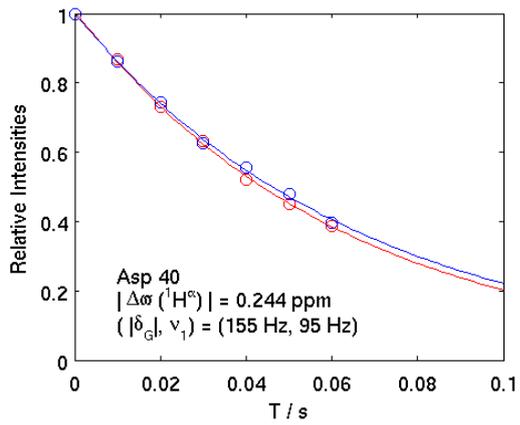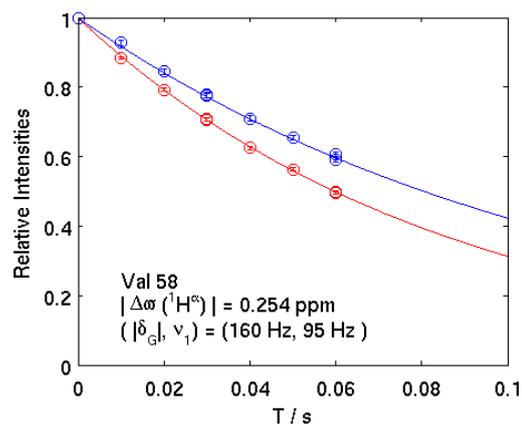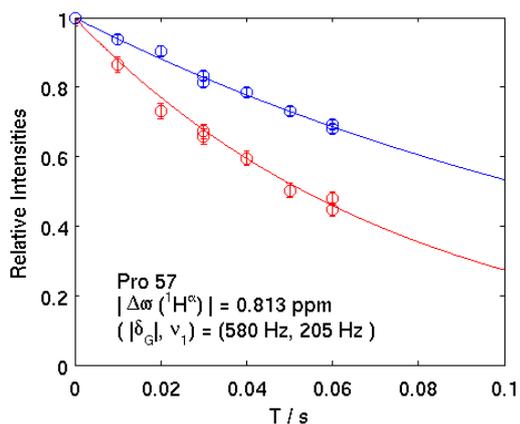
[a] Difference in $R_{1\rho}^{\pm}$ decay profiles could not be observed using optimized $(|\delta_{G}|, \nu_{1})$ values. Complete $R_{1\rho}$ vs δ_{G} profiles were recorded at several ω_{1} values to determine the optimal $(|\delta_{G}|, \nu_{1})$ values experimentally. Values of $(|\delta_{G}|, \nu_{1})=(200\text{Hz},100\text{Hz})$ were chosen to produce the $R_{1\rho}^{\pm}$ decay curves in the figure.

Each data point was obtained in approximately 6 minutes (room temperature probe-head), providing excellent signal-to-noise (≈ 1 mM sample).

Table T2. 24 residues in the A39V/N53P/V55L Fyn SH3 domain for which $^1H^\alpha$ $\Delta\varpi$ (ϖ_E - ϖ_G) values were measured using the CPMG relaxation dispersion experiment as described in ref 4. $|\Delta\varpi|$ values from CPMG experiments along with signs obtained from off-resonance $R_{1\rho}$ measurements using the pulse sequence of Figure 1 are indicated. Sign information could not be obtained in many cases where $|\Delta\varpi|<$ 0.25 ppm (indicated by 'no sign'). In the case considered here the excited state corresponds to a folding intermediate[6].

Residue	$\Delta\varpi$ (Extracted) / ppm	t value[a]
Als 56	- 0.69 ± 0.04	24
Val 58	+ 0.25 ± 0.02	18
Glu 5	- 0.93 ± 0.05	15
Pro 57	+ 0.81 ± 0.05	12
Leu 7	+ 0.46 ± 0.03	12
Pro 53	- 0.30 ± 0.03	11
Ala 6	- 1.26 ± 0.08	10
Ile 50	+ 0.40 ± 0.03	10
Lys 25	- 0.34 ± 0.02	10
Tyr 49	+ 0.34 ± 0.02	10
Thr 47[b]	- 0.17 ± 0.02	9
Phe 4	- 0.30 ± 0.02	8
Tyr 8	- 0.25 ± 0.02	7
Leu 3	+ 0.29 ± 0.02	6
Ser 52[b]	- 0.22 ± 0.02	6
Asp 9	+ 0.37 ± 0.03	4
Glu 38	+ 0.37 ± 0.02	3
Asp 16	- 0.14 ± 0.03	3
Tyr 54	+ 0.13 ± 0.02	3
Arg 40	0.24 ± 0.02 (no sign)	3
Lys 22	0.19 ± 0.02 (no sign)	2
Glu 33	0.16 ± 0.01 (no sign)	2
Leu 18	0.14 ± 0.02 (no sign)	2
His 21	0.14 ± 0.02 (no sign)	2

[a] See legend 'a' for table T1.

[b] Note, while the errors of $\Delta\varpi$ values for serine and threonine residues are higher as discussed in detail previously[4], the signs can still be extracted accurately using the present off-resonance $^1H^\alpha$ $R_{1\rho}$ experiment.

It is of interest to note that signs were obtained for smaller $|\Delta\varpi|$ values in the case of the Abp1p system than for the A39V/N53P/V55L Fyn SH3 domain, reflecting the different exchange parameters that are operative in each case; (p_E,k_{ex}) = (6%, 300s^{-1}) and (1.4%,780s^{-1}) for Abp1p and A39V/N53P/V55L Fyn SH3, respectively.

Figure S3. The importance of optimizing values of $\Delta(\delta_G, \omega_I)$ used in $R_{1\rho}$ experiments. Values of $\Delta(\delta_G, \omega_I)$ have been optimized (see eqs 3 and 4) for Ala56 (top) and Pro57 (bottom) of the A39V/N53P/V55L Fyn SH3 domain (left panels) using exchange parameters, $k_{ex} = 780$ s^{-1} and $p_E = 1.4\%$ (20°C), along with values of $^1H^{\alpha}$ $|\Delta\varpi| = 0.687$ ppm (550 Hz at 800 MHz) and $|\Delta\varpi| = 0.813$ ppm (650 Hz at 800 MHz) for residues 56 and 57, respectively (values obtained from analysis of CPMG data sets). Optimized $(|\delta_G|, \nu_I)$ values are (485 Hz, 180Hz) for Ala56 and (585Hz, 195Hz) for Pro57. $R_{1\rho}$ decay curves calculated using the optimized values are shown in solid lines (right panels) for Ala56 (top) and Pro57 (bottom), with corresponding values calculated using $|\delta_G| = |\delta\varpi|$ and $\nu_1 = 50$ Hz, dashed lines. Red (blue) decay curves are calculated for the spin-lock field positioned downfield (upfield) of the resonance position of the probe in the ground state. It is clear that larger differences in $R_{1\rho}$ decay curves are obtained with optimized values, leading to more accurate determinations of signs.
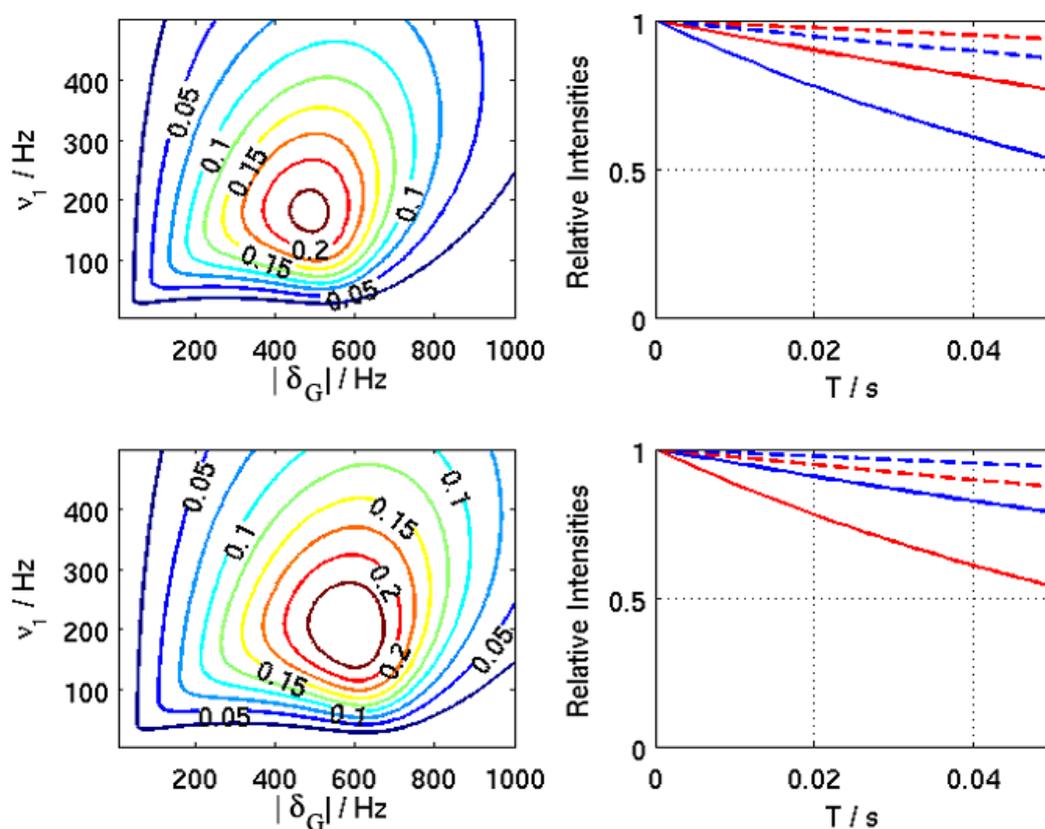
(1)   Korzhnev, D. M.; Orekhov, V. Y.; Kay, L. E. *J Am Chem Soc* **2005**, *127*, 713-721.
(2)   Shaka, A. J.; Keeler, J.; Frenkiel, T.; Freeman, R. *J. Magn. Reson.* **1983**, *52*, 335-338.
(3)   Vallurupalli, P.; Hansen, D. F.; Kay, L. E. *Proc Natl Acad Sci U S A* **2008**, *105*, 11766-11771.
(4)   Lundstrom, P.; Hansen, D. F.; Vallurupalli, P.; Kay, L. E. *J Am Chem Soc* **2009**, *131*, 1915-1926.
(5)   Zar, J. H. *Biostatistical Analysis*, 2nd edition, **1984,** Prentice-Hall, New Jersey
(6)   Neudecker, P.; Zarrine-Afsar, A.; Choy, W. Y.; Muhandiram, D. R.; Davidson, A. R.; Kay, L. E. *J Mol Biol* **2006**, *363*, 958-976.