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Article

Accuracy of optimized chemical-exchange parameters derived by fitting CPMG R_2 dispersion profiles when $R_2^{0a} \neq R_2^{0b}$

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Abstract

The transverse relaxation rate, R_2, measured as a function of the effective field (R_2 dispersion) using a Carr-Purcell-Meiboom-Gill (CPMG) pulse train, is well suited to detect conformational exchange in proteins. The dispersion data are commonly fitted by a two-site (sites a and b) exchange model with four parameters: the relative population, p_a, the difference in chemical shifts of the two sites, $\delta\omega$, the correlation time for exchange, τ_{ex}, and the intrinsic relaxation rate (i.e., transverse relaxation rate in the absence of chemical exchange), R_2^0. Although the intrinsic relaxation rates of the two sites, R_2^{0a} and R_2^{0b}, can differ, they are normally assumed to be the same (i.e., $R_2^{0a} = R_2^{0b} = R_2^0$) when fitting dispersion data. The purpose of this investigation is to determine the magnitudes of the errors in the optimized exchange parameters that are introduced by the assumption that $R_2^{0a} = R_2^{0b}$. In order to accomplish this goal, we first generated synthetic constant-time CPMG R_2 dispersion data assuming two-site exchange with $R_2^{0a} \neq R_2^{0b}$, and then fitted the synthetic data assuming two-site exchange with $R_2^0 = R_2^{0a} = R_2^{0b}$. Although all the synthetic data generated assuming $R_2^{0a} \neq R_2^{0b}$ were well fitted (assuming $R_2^{0a} = R_2^{0b}$), the optimized values of p_a and τ_{ex} differed from their true values, whereas the optimized values of $\delta\omega$ values did not. A theoretical analysis using the Carver–Richards equation explains these results, and yields simple, general equations for estimating the magnitudes of the errors in the optimized parameters, as a function of ($R_2^{0a} - R_2^{0b}$).

Introduction

Local conformational changes in proteins on the ms- μs time scale often cause an increase the rate of transverse spin relaxation, R_2, via the chemical exchange mechanism. Determination of chemical exchange parameters, such as lifetimes, populations and chemical shifts of the exchanging species is of interest in order to characterize the kinetics and thermodynamics of a conformational transition that is relevant to function (Palmer et al., 2001; Korzhnev et al., 2004b). The exchange parameters are often derived from the measurements of R_2 dispersion, where R_2 is measured as a function of the effective field strength (ν_{CP}) in a CPMG experiment (Orekhov et al., 1994; Ishima et al., 1998; Loria et al., 1999; Mulder et al., 2001). In particular, an efficient dispersion experiment, in which one reference 2D spectrum and a series of 2D spectra with various delays between CPMG pulses, $2\tau_{CP}$, applied for a constant relaxation period, is well

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suited for protein studies (Mulder et al., 2001; Skrynnikov et al., 2001; Tollinger et al., 2001; Ishima and Torchia, 2003).

The simplest and most widely used model of the chemical exchange involves two sites, a and b. In this case, R_2 depends upon five parameters: (1) the relative population of site *a*, p_a; (2) the exchange correlation time, τ_{ex}; (3) the difference in chemical shifts of the two sites, $\delta\omega$; and (4, 5) the intrinsic relaxation rates without chemical exchange, R_2^{0a} and R_2^{0b}. Unless exchange is in the slow limit and two resolved signals are detected, it is usual to assume that $R_2^{0a} = R_2^{0b} = R_2^0$. However, in general R_2^{0a} is not equal to R_2^{0b}. In studies of proteins, significant differences between R_2^{0a} and R_2^{0b} would be expected when exchanging species are (a) folded and unfolded states of a protein, (b) a monomer in equilibrium with an oligomer, and (c) a ligand free and bound to a large protein.

Early theoretical work showed that the $R_2^{0a} = R_2^{0b}$ assumption had little impact upon the analysis of exchange involving small molecules because $\delta R_2^0 = (R_2^{0a} - R_2^{0b})$ was very small (Woessner, 1960; Vold and Chan, 1972). In contrast, Davis et al. (1994) showed that δR_2^0 of a small molecule ligand, exchanging between free and protein-bound states, was significant. In this case proper fitting of the dispersion data required independent determinations of R_2^{0a} and R_2^{0b}. Recently, in a systematic study of the very slow exchange ($\tau_{ex} > 100$ ms) between unfolded and folded states of an SH3 domain, the effect of the difference between R_2^{0a} and R_2^{0b} was estimated to be negligible when $p_a >> p_b$ (Tollinger et al., 2001; Korzhnev et al., 2004a).

From the above remarks it is clear that using the $\delta R_2^0 = 0$ assumption to fit dispersion data can introduce either insignificant or substantial errors in optimized exchange parameters, depending upon circumstances. Therefore our purpose is to obtain a general quantitative understanding about errors in optimized exchange parameters that are introduced by assuming that $R_2^{0a} = R_2^{0b}$ when fitting experimental dispersion curves. We do this by first generating synthetic dispersion data using the relaxation matrix approach of Jen (1978) to numerically calculate R_2 as a function of ν_{CP} during a CPMG pulse train. We next fit the synthetic dispersion data calculated with $R_2^{0a} \neq R_2^{0b}$ (i.e. using five parameters) by the same numerical approach, but assuming that $R_2^{0a} = R_2^{0b}$ (i.e. using four parameters). We then compare the optimized parameters with their true values, and explain the errors in the parameters using the Carver–Richards equation (Carver and Richards, 1972; Jen, 1978; Davis et al., 1994). The Carver–Richards equation is also used to develop simple general expressions for the errors in the optimized parameters as a function of δR_2^0.

Methods

Generation of synthetic R_2 dispersion data

Transverse magnetization, I(t), during a constant time CPMG pulse train was generated by a numerical solution of the Bloch–McConnell equations (McConnell, 1958) using the matrix method of Jen (1978). I(t) for a series of ν_{CP} values was calculated using 5 parameters: (1) the population of site *a*, p_a; (2) the correlation time for exchange, τ_{ex}; (3) the difference in chemical shifts between sites a and b, $\delta\omega$; (4, 5) the intrinsic transverse relaxation rates of sites a and b respectively, R_2^{0a} and R_2^{0b}. Off-resonance effects were assumed to be negligible. For fast and intermediate exchange, R_2 was calculated as $-\log(I(T_{cp})/I(0))/T_{cp}$ where $I(0) = 1$ and T_{cp} is the constant CPMG relaxation period, assumed to be 200 ms. For slow exchange, R_2 was calculated according to $-\log(I^a(T_{cp})/I^a(0))/T_{cp}$ where $I^a(0) = p_a$. MATLAB software (Mathworks Inc, MA) was used for the calculations.

R_2 dispersion curve fitting

The synthetic R_2 dispersion curves generated using five parameters ($R_2^{0a} \neq R_2^{0b}$) were fitted using four parameters ($R_2^{0a} = R_2^{0b} = R_2^0$). The optimized exchange parameters, $R_2^0, \delta\omega, p_a$, and τ_{ex}, were calculated by minimizing the quality function, Q^2, where

$$Q^2 = \sum_i \left(\frac{R_2^{i,\mathrm{syn}} - R_2^{i,\mathrm{fit}}}{R_2^{i,\mathrm{syn}}} \right)^2 \qquad (1)$$

Here, $R_2^{i,\mathrm{syn}}$ and $R_2^{i,\mathrm{fit}}$, are synthetic and fitted R_2 values of *i*-th ν_{CP} value, respectively. Q is the value

of the total rms fractional difference of $R_2^{i,\mathrm{fit}}$ from $R_2^{i,\mathrm{syn}}$, and is a measure of the quality of the four parameter fit ($R_2^{0a} = R_2^{0b}$) to the synthetic data generated with five parameters ($R_2^{0a} \neq R_2^{0b}$). To ensure that the global Q^2 minimum was found, Q^2 minimization was repeated using a range of initial parameters, in both slow and fast exchange regimes.

The Monte Carlo method was used to estimate the uncertainties of the four optimized parameters due to random noise in the R_2 data. One hundreds "noisy" synthetic R_2 dispersion data sets were generated by adding random Gaussian noise, with rms amplitude $\Delta R_2^{i,\mathrm{err}} = 2\%$, to each R_2 value. The parameters optimized by minimizing Q^2 (Equation (1)) were used as initial parameters to fit each of the 30 noisy data sets. For each data set, four optimized exchange parameters were obtained by minimizing the target function.

$$\chi^2 = \sum_i \left(\frac{R_2^{i,\mathrm{syn}} - R_2^{i,\mathrm{fit}}}{\Delta R_2^{i,\mathrm{err}}} \right)^2 \tag{2}$$

The uncertainty of each exchange parameter was calculated as the standard deviation of the 100 optimized values of the parameter obtained from the Monte Carlo fits.

Theoretical analysis of errors in optimized parameters

We used the Carver–Richards equation (Carver and Richards, 1972) as corrected by (Jen, 1978; Davis et al., 1994) in order to understand the observed differences between optimized parameters derived by minimizing Q (i.e. fits obtained assuming $R_2^{0a} = R_2^{0b}$) and the parameters used to generate the synthetic R_2 values (calculated assuming $R_2^{0a} \neq R_2^{0b}$). The Carver–Richards equation can be written as follows:

$$R_2 = (R_2^{0a} + R_2^{0b} + 1/\tau_{ex})/2 - (1/2\tau_{CP}) \ln \lambda^+ \tag{3.1}$$

$$\ln \lambda^+ = \ln[(D_+ \cos h^2 \xi - D_- \cos^2 \eta)^{1/2} + (D_+ \sin h^2 \xi + D_- \sin^2 \eta)^{1/2}] \tag{3.2}$$

$$D_\pm = 1/2 \left[\pm 1 + (\psi + 2(\delta\omega)^2)/(\psi^2 + \zeta^2)^{1/2} \right] \tag{3.3}$$

$$\xi = (\tau_{CP}/2\sqrt{8}) \left[+\psi + (\psi^2 + \zeta^2)^{1/2} \right]^{1/2} \tag{3.4}$$

$$\eta = (\tau_{CP}/2\sqrt{8}) \left[-\psi + (\psi^2 + \zeta^2)^{1/2} \right]^{1/2} \tag{3.5}$$

$$\psi = (\delta R_2^0 - \delta p/\tau_{ex})^2 - (\delta\omega)^2 + 4(p_a p_b/\tau_{ex}^2) \tag{3.6}$$

$$\zeta = 2(\delta\omega)(\delta R_2^0 - \delta p/\tau_{ex}) \tag{3.7}$$

Here, $\tau_{cp} (= 1/4\nu_{CP})$ is the half duration between CPMG 180° pulses, and $\delta X = X_a - X_b$.

Examination of Equation (3) reveals that R_2 calculated using five parameters ($p_a, \delta\omega, \tau_{ex}, R_2^{0a}, R_2^{0b}$) equals R_2 calculated using four parameters ($p_a^*, \delta\omega*, \tau_{ex}^*, R_2^{0*}$) when the following conditions are satisfied.

$$2R_2^{0*} + 1/\tau_{ex}^* = R_2^{0a} + R_2^{0b} + 1/\tau_{ex} \tag{4}$$

$$\delta p^*/\tau_{ex}^* = \delta R_2^0 - \delta p/\tau_{ex} \tag{5}$$

$$p_b^* p_a^*/(\tau_{ex}^*)^2 = p_b p_a/(\tau_{ex})^2 \tag{6}$$

$$\delta\omega^* = \delta\omega \tag{7}$$

In order to establish the range of validity of Equations (4–7), we first note that when $\delta R_2^0 \tau_{ex} << 1$, Jen's equation for R_2 in the fast pulsing (strong effective field) limit $R_2(\nu_{CP} \to \infty)$ (Jen, 1978; Davis et al., 1994) can be simplified by retaining terms less than second order in $\delta R_2^0 \tau_{ex}$ and written as

$$R_2(\nu_{CP} \to \infty) = R_2^{0*} \approx p_a R_2^{0a} + p_b R_2^{0b} - p_a p_b \tau_{ex} (\delta R_2^0)^2 \tag{8}$$

Replacing R_2^{0*} in Equation (4) by the right-hand side of Equation (8), and then adding and subtracting Equations (4) and (5) and retaining terms that are less than second order in $\delta R_2^0 \tau_{ex}$, one obtains the following relationships between the exchange parameters

$$\tau_{ex}^* = \tau_{ex}(1 - \delta p \delta R_2^0 \tau_{ex})^{-1} \tag{9}$$

$$p_a^* = p_a(1 - \delta R_2^0 \tau_{ex})/(1 - \delta p \delta R_2^0 \tau_{ex}) \tag{10.1}$$

$$p_b^* = p_b(1 + \delta R_2^0 \tau_{ex})/(1 - \delta p \delta R_2^0 \tau_{ex}) \qquad (10.2)$$

Replacing τ_{ex}^* and p_a^* in Equation (6) by right-hand sides of Equations (9) and (10) shows that Equation (6) is valid when $(\delta R_2^0 \tau_{ex})^2 << 1$. Therefore, this analysis, based upon the Carver–Richards equation, predicts that, when $|\delta R_2^0| > 0$, excellent fits of R_2 dispersion data will be obtained assuming that $R_2^{0a} = R_2^{0b}$, provided that $(\delta R_2^0 \tau_{ex})^2 << 1$. In addition, when this inequality is satisfied and the Carver–Richards equation is accurate, Equations (4), (9), and (10) provide simple, yet quantitative, predictions of the fractional errors in optimized values of $\delta\omega, \tau_{ex}, p_a$ and p_b derived from four parameter fits.

For completeness, we note that in the fast exchange limit, $1/\tau_{ex} >> \delta\omega, \delta R_2^0$, the Carver–Richards equation (Equation (3)) becomes (Palmer et al., 2001)

$$R_2 = p_a R_2^{0a} + p_b R_2^{0b} + (p_a p_b (\delta\omega)^2 \tau_{ex}) \times [1 - (\tau_{ex}/\tau_{CP}) \tanh(\tau_{CP}/\tau_{ex})] \qquad (11)$$

It is clear from Equation (11) that in the fast exchange limit, only three parameters, $R_2^0 = p_a R_2^{0a} + p_b R_2^{0b}, \Phi = p_a p_b (\delta\omega)^2$, and τ_{ex} can be derived from fitted dispersion data. In addition, because R_2 depends on only the population-weighted average of R_2^{0a} and R_2^{0b}, parameter optimization is independent of δR_2^0.

In the subsequent presentation we employ the three sets of parameters that are distinguished by the following superscripts: (a) the superscript "syn" identifies the five parameters $(p_a^{syn}, \delta\omega^{sim}, \tau_{ex}^{syn}, R_2^{0a,syn}, R_2^{0b,syn})$ that are used to generate the synthetic R_2 dispersion data; (b) the superscript "fit" is applied to the four optimized parameters $(p_a^{fit}, \delta\omega^{fit}, \tau_{ex}^{fit}, R_2^{0,fit})$ that are derived by fitting the synthetic data according to Equation (1); (c) the superscript "*" identifies the four parameters $(p_a^*, \delta\omega^*, \tau_{ex}^*, R_2^{0,*})$ that are obtained from Equations (7) to (10).

Results and discussion

Generation of relaxation dispersion data in intermediate exchange

We generated nine transverse relaxation dispersion data sets using the parameters listed in Table 1. For all nine data sets, $\delta\omega^{syn}\tau_{ex}^{syn} = 1.57$, implying that exchange is on the intermediate time scale.

Table 1. Optimized parameters obtained assuming the $R_2^{0a} = R_2^{0b} = R_2^0$ model to fit intermediate exchange data*

Original parameters	Optimized values and percentage differences of the values from the original parameters used for the data generation						
	p_b^{fit}	Δp_b^{**} (%)	$\delta\omega^{fit}/2\pi$ (Hz)	τ_{ex}^{fit} (ms)	$\Delta\tau_{ex}^{**}$ (%)	$R_2^{0,fit}$ (s^{-1})	Q^{***}
$p_b^{syn} = 0.3$****							
$\delta R_2^{0,syn} = 0\ s^{-1}$	0.300	0.0	50.0	5.00	0.0	20.0	6.69×10^{-6}
$\delta R_2^{0,syn} = 10\ s^{-1}$	0.321	7.0	50.0	5.09	1.8	16.9	5.09×10^{-4}
$\delta R_2^{0,syn} = 15\ s^{-1}$	0.332	11.0	50.0	5.14	2.8	15.3	8.28×10^{-4}
$p_b^{syn} = 0.2$							
$\delta R_2^{0,syn} = 0\ s^{-1}$	0.200	0.0	50.0	5.00	0.0	20.0	7.65×10^{-6}
$\delta R_2^{0,syn} = 10\ s^{-1}$	0.218	8.0	50.0	5.15	3.0	17.9	3.49×10^{-4}
$\delta R_2^{0,syn} = 15\ s^{-1}$	0.225	11.2	50.0	5.22	4.4	16.8	5.69×10^{-4}
$p_b^{syn} = 0.1$							
$\delta R_2^{0,syn} = 0\ s^{-1}$	0.100	0.0	50.0	5.00	0.0	20.0	7.28×10^{-6}
$\delta R_2^{0,syn} = 10\ s^{-1}$	0.109	9.0	50.0	5.20	4.0	19.0	1.99×10^{-4}
$\delta R_2^{0,syn} = 15\ s^{-1}$	0.114	14.0	50.0	5.31	6.2	18.4	3.24×10^{-4}

*Parameter optimizations were performed for R_2 dispersion data generated with the parameters $\delta\omega^{syn} = 50$ Hz, $\tau_{ex}^{syn} = 5$ ms, and $R_2^{a,syn} = 20\ s^{-1}$ and the p_b^{syn} and $\delta R_2^{0,syn} (= R_2^{0a} - R_2^{0a})$ values listed in the left most column in the Table.

**The percentage differences of the fit parameters from the parameters used to generate the data for p_b and τ_{ex} are Δp_b and $\Delta\tau_{ex}$, respectively. The percentage difference was not listed for $\delta\omega$ because it was less than 0.1% in every case.

***Q^2 given by Equation (1) was used as the target function of minimization.

****$p_b^{syn} = 1 - p_a^{syn}$. The minor population, p_b^{syn}, is listed because the percentage difference from the true value is larger for p_b^{syn} than p_a^{syn}.

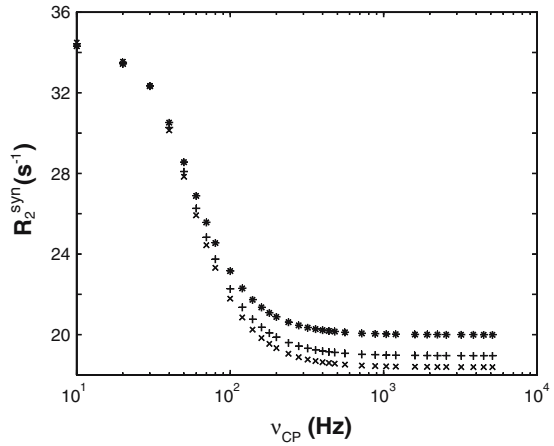

Figure 1. Synthetic R_2 dispersion data, calculated for the case of intermediate exchange ($\tau_{ex}^{syn}\delta\omega^{syn} = 1.57$), plotted as a function of the effective field strength, ν_{CP}. A matrix solution of the two-site Bloch–McConnell equations (Jen, 1978) was used to calculate R_2 using MATLAB together with the following parmeters: $p_a^{syn} = 0.9, \tau_{ex}^{syn} = 5$ ms, $\delta\omega^{syn}/2\pi = 50$ Hz, $R_2^{0a,syn} = 20$ s^{-1}, and $R_2^{0b,syn} = 20(*)$, 10 (+), and 5 (×) s^{-1}.

Three of the synthetic data sets generated with $R_2^{0b,syn} = 20, 10$, and 5 s^{-1} ($\delta R_2^{0,syn} = 0, 10, 15$ s^{-1}, respectively) are shown in Figure 1. Figure 1 reveals that in the strong field limit, $\nu_{CP} \rightarrow \infty$, the plateau value of R_2, R_2^0, decreases as $R_2^{0b,syn}$ decreases (i.e., as $\delta R_2^{0,syn}$ increases) because R_2^0 is a population-weighted average of R_2^{0a} and R_2^{0b}, as described in Equation (8). On the other hand, in the weak field limit, $\nu_{CP} \rightarrow 0$, R_2 is independent of $R_2^{0b,syn}$. This latter observation is predicted by the Carver–Richards equation in the weak field limit (Jen, 1978). In this limit, R_2^a is equal to the real part of the lowest eigenvalue of the Bloch–McConnell relaxation matrix (Davis et al., 1994; Cavanagh et al., 1996). When $\delta\omega^2 >> (1/\tau_{ex})^2$, $(\delta R_2^0)^2$, a straightforward calculation yields

$$R_2^a(\nu_{CP} \rightarrow 0) \approx R_2^{0a} + p_b/\tau_{ex} + p_a p_b \times (\delta R_2^0 - \delta p/\tau_{ex})/(\delta\omega^2\tau_{ex}^2)) \quad (12)$$

The expression for $R_2^b(\nu_{CP} \rightarrow 0)$ is obtained by interchanging subscripts 'a' and 'b' in Equation (12). Inserting the values of the exchange parameters used to generate the curves in Figure 1 into Equation (12), $R_2^a(\nu_{CP} \rightarrow 0)$ changes by less than 2% as $R_2^{0b,syn}$ decreases from 20 to 5 s^{-1}. This predicted result is in agreement with the synthetic data plotted in Figure 1.

Parameter optimization using the $R_2^{0a} = R_2^{0b}$ model in intermediate exchange

Using the $R_2^{0a} = R_2^{0b}$ model, the four parameters $(p_a^{fit}, \delta\omega^{fit}, \tau_{ex}^{fit}, R_2^{0,fit})$ were optimized by fitting the synthetic dispersion data in Table 1 that were generated using 5 parameters $(p_a^{syn}, \delta\omega^{syn}, \tau_{ex}^{syn}, R_2^{0a,syn}, R_2^{0b,syn})$. The parameter optimizations were performed by minimizing Q^2 defined in Equation (1). Q is the square-root of the sum of the fractional differences between $R_2^{0,syn}$ and $R_2^{0,fit}$. Data generated using $R_2^{0a} = R_2^{0b}$ were fitted to test that the optimization yielded optimized parameters that equaled those used to generate the synthetic data. The optimized parameters and the Q values for all intermediate exchange date fitted are listed in Table 1.

All Q values listed in Table 1 are less than 6×10^{-4}, which implies that the average value of $(R_2^{0,syn} - R_2^{0,fit})/R_2^{0,syn}$ is less than 1.8×10^{-5} (= 0.0018%). Because this number is much less than the R_2 error in dispersion experiments (typically >1%), a four-parameter fit with $R_2^{0a} = R_2^{0b} = R_2^0$ will, in practice, fit a dispersion data set as well as a five-parameter fit. Note that in Table 1, the Q values for synthetic data generated assuming that $R_2^{0a} = R_2^{0b}$ (i.e., four parameters were used to generate the synthetic data) are at least 30 times smaller than Q values for synthetic data generated using five parameters, i.e., $R_2^{0a} \neq R_2^{0b}$. In addition, the optimized parameters are in essentially perfect agreement with the parameters used to generate the synthetic data. These results confirm that the optimization was correctly performed.

Table 1 shows that the optimized p_b^{fit} and τ_{ex}^{fit} values increasingly differ from the parameters used to generate the synthetic data as the $\delta R_2^{0,syn}$ increases, a result that is clearly depicted in Figure 2. In addition, it is evident that when the sign of $\delta R_2^{0,syn}$ reverses, so do the signs of the deviations of optimized parameters from their true values because, as is predicted by Equations 8–10, the sign of the deviations depends on the sign of δR_2^0. In contrast, the optimized value of $\delta\omega^{fit}$ is independent of $\delta R_2^{0,syn}$ (Table 1). In spite of the fact that nearly perfect fits of relaxation dispersion data are obtained using the four-parameter model, the optimized parameters p_b^{fit} and τ_{ex}^{fit} deviate from their correct values by 14% and 6%, respectively, when $\delta R_2^{0,syn} = 15$ s^{-1}.

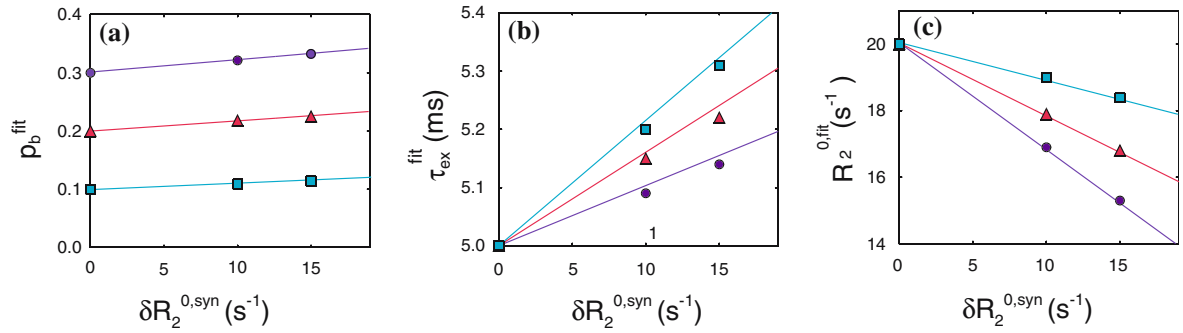

Figure 2. Plots of optimized exchange parameters (a) p_b^{fit}, (b) τ_{ex}^{fit}, and (c) $R_2^{0,fit}$ obtained by fitting intermediate-exchange R_2 data, generated assuming five parameters, with a four-parameter model. The synthetic data were generated using the following parameters: $p_b^{syn} = 0.1, 0.2$, and 0.3, $\tau_{ex}^{syn} = 5$ ms, $\delta\omega^{syn}/2\pi = 50$ Hz, $R_2^{0a,syn} = 20\ s^{-1}$, $\delta R_2^0 = 0$, 10, and 15 s^{-1}. The optimized values at $p_b^{syn} = 0.1$, 0.2, and 0.3 are plotted using □ (cyan), Δ (red), and ○ (purple), respectively. The parameter values predicted by Equations (10), (9), and (8) for p_b^{fit}, τ_{ex}^{fit}, and $R_2^{0,fit}$, respectively, are plotted as three colored lines, corresponding to $p_b^{syn} = 0.1$ (cyan), 0.2 (red), and 0.3 (purple).

Table 2. Percentage uncertainities of the optimized exchange parameters listed in Table 1 due to a rms 2% uncertainty in the synthetic R_2 data

Initial parameters	Optimized parameter uncertainties and normalized χ^2 values				
	p_b^{fit} (%)	$\delta\omega^{fit}/2\pi$ (%)	τ_{ex}^{fit} (%)	$R_2^{0,fit}$ (%)	χ^2/N^*
$p_b^{syn} = 0.3$					
$\delta R_2^{0,syn} = 0\ s^{-1}$	2.6	2.2	7.2	0.47	0.98
$\delta R_2^{0,syn} = 10\ s^{-1}$	2.5	1.8	6.8	0.44	1.03
$\delta R_2^{0,syn} = 15\ s^{-1}$	2.4	1.8	6.3	0.57	0.96
$p_b^{syn} = 0.2$					
$\delta R_2^{0,syn} = 0\ s^{-1}$	3.5	3.2	11.1	0.43	1.01
$\delta R_2^{0,syn} = 10\ s^{-1}$	3.7	3.2	11.0	0.42	1.05
$\delta R_2^{0,syn} = 15\ s^{-1}$	3.2	3.1	10.5	0.45	1.00
$p_b^{syn} = 0.1$					
$\delta R_2^{0,syn} = 0\ s^{-1}$	19.0	10.4	35.4	0.46	0.98
$\delta R_2^{0,syn} = 10\ s^{-1}$	17.4	8.0	38.4	0.43	1.03
$\delta R_2^{0,syn} = 15\ s^{-1}$	15.7	7.6	37.8	0.46	0.96

*Uniform 2% rms uncertainities were assumed for the relaxation dispersion data. Uncertainities of the optimized values were estimated as the standard deviations of the parameter distributions derived from the Monte-Carlo calculations. The target function used for the minimization in each trial is given by χ^2 in Equation (2).

In order to compare the magnitude of these errors in optimized parameters with parameter uncertainties introduced by experimental error, the parameter optimization was performed incorporating a random uncertainty of 2% (rms) in all the synthetic R_2 data. The optimized parameters were obtained by minimizing the χ^2 target function (Equation (2)) and the parameter uncertainties were derived from the Monte-Carlo approach described in Methods. The parameter uncertainties and the average values of the normalized χ^2 functions, χ^2/N, are listed in Table 2. The values of χ^2/N, listed in Table 2, are close to unity, indicating that the optimizations are consistent with the rms noise and small Q values listed in the Table 1. The uncertainties of the optimized parameters due to the 2% rms random uncertainty in R_2, are in the ranges of 2–20% and 6–40% for p_b^{fit} and τ_{ex}^{fit}, respectively, and increase as p_b^{syn} decreases (Table 2). This latter result is explained by the reduction of the amplitude of the exchange contribution to R_2 as p_b^{syn} decreases, which causes

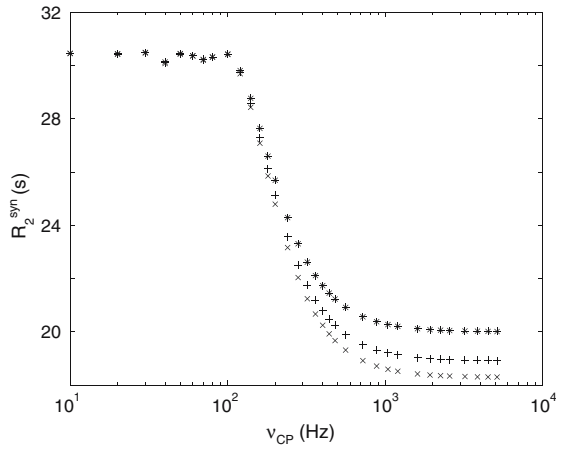

Figure 3. Synthetic R_2 dispersion data, calculated in the limit of slow exchange, plotted as a function of the effective field strength, ν_{CP}. A matrix solution of the two-site Bloch–McConnell equations (Jen, 1978) was used to calculate R_2 using MATLAB together with the following parameters: $p_a^{syn} = 0.9, \tau_{ex}^{syn} = 10$ ms, $\delta\omega^{syn}/2\pi = 200$ Hz, $R_2^{0a,syn} = 20\ s^{-1}$, and $R_2^{0b,syn} = 20(*)$, 10 (+), and 5 (×) s^{-1}.

an increase the fractional uncertainty in the exchange contribution to R_2.

Comparison of Table 1 with Table 2 clearly indicates that the error in p_b^{fit} introduced by four-parameter fitting is larger than the uncertainty in p_b^{fit} which arises from the 2% rms uncertainties in the R_2 values when $p_b^{fit} > 0.1$. In contrast, the error in τ_{ex}^{fit} introduced by the four-parameter fit is smaller than the uncertainty caused by the 2% rms uncertainty in R_2. We note that the experimental uncertainties in R_2 of less than 2% have been reported for dispersion profiles recorded using a low temperature probe (Ishima and Torchia, 2005). In such cases the errors in exchange parameters caused by the $R_2^{0a} = R_2^{0b}$ assumption will be more significant than indicated by the results in Tables 1 and 2.

Comparison of parameters predicted by theory with those derived from four-parameter fits of synthetic R_2 data

The fitted optimized parameters (p_a^{fit}, τ_{ex}^{fit}, and $R_2^{0,fit}$) are plotted as functions of δR_2^0 in Figure 2 along with curves depicting the theoretical functional dependence of the fitted parameters on δR_2^0, predicted by Equations 8–10. The plots show that there is excellent agreement between the predicted and fitted values of the optimized parameters. For all simulated data in Table 1, $\delta R_2 \tau_{ex} < 0.075$. In the limit that $\delta R_2 \tau_{ex} << 1$, Equations (9) and (10.2) predict that differences between the true and fitted values of τ_{ex} and p_b are proportional to δR_2, as observed in Figure 2. Furthermore, the linear decrease of $R_2^{0,fit}$ as δR_2 increases, Figure 2(c), is predicted by Equation (8). Finally, the equality of $\delta\omega^{syn}$ with $\delta\omega^{fit}$ for all values of δR_2 is in accord with Equation (7). Therefore in the intermediate exchange regime where the condition $\delta R_2 \tau_{ex} << 1$ is expected to be satisfied, Equations (7)–(10) predict reliable values of the errors in optimized parameters caused by the $R_2^{0a} = R_2^{0b}$ assumption. Of course, approximate values of $\tau_{ex}, p_b, \delta\omega$, and δR_2 must be available in order to use the theoretical equations to predict such errors. Approximate values of these parameters will usually be available based upon estimated (by calculation or experiment) rotational correlation times of the molecules under study and of values of optimized parameters obtained from preliminary four-parameter fits.

Parameter optimization using the $R_2^{0a} = R_2^{0b}$ model in slow exchange

Using the $R_2^{0a} = R_2^{0b}$ model, the four parameters ($p_a^{fit}, \delta\omega^{fit}, \tau_{ex}^{fit}, R_2^{0,fit}$) were optimized by fitting to the synthetic dispersion data generated using 5 parameters ($p_a^{syn}, \delta\omega^{syn}, \tau_{ex}^{syn}, R_2^{0a,syn}, R_2^{0b,syn}$) in the limit of slow exchange, $\delta\omega^{syn}\tau_{ex}^{syn} = 12.6$ (Figure 3 and Table 3). Here, $\delta\omega^{syn}/2\pi$ (200 Hz) and τ_{ex}^{syn} (10 ms) are respectively four times and two times larger than those used in the case of intermediate exchange to satisfy the slow exchange condition. The parameter optimizations were performed by minimizing Q^2 defined in Equation (1). The Q values listed in Table 3 for fits to the data generated assuming that $R_2^{0a} = R_2^{0b}$ are the same order as those in Table 1, demonstrating that the optimization was properly performed. The maximum Q values listed in Table 3 for the fits of the $R_2^{0a} \neq R_2^{0b}$ data were ca. 6×10^{-3}, about 10 times larger than those listed in Table 1 for the intermediate exchange case. Nevertheless, the small Q values listed in Table 3 imply that the average value of $(R_2^{0,syn} - R_2^{0,fit})/R_2^{0,syn}$ is less than 1.8×10^{-4} (= 0.018%) for the slow exchange data. These fractional differences between synthetic and fitted R_2 values are far less than the fractional random uncertainties reported for measured R_2 values (Korzhnev et al., 2004a; Yao et al., 2004; Ishima and Torchia, 2005); therefore, in practice, a

Table 3. Optimized parameters obtained assuming the $R_2^{0a} = R_2^{0b} = R_2^0$ model to fit intermediate exchange data*

Initial parameters	Optimized values and percentage uncertainities						
	p_b^{fit} value	Δp_b^{fit} (%)	$\delta\omega^{fit}/2\pi$ (Hz)	τ_{ex}^{fit} (ms)	$\Delta\tau_{ex}^{fit}$ (%)	$R_2^{0,fit}$ (s^{-1})	Q
$p_b^{syn} = 0.3$							
$\delta R_2^{0,syn} = 0\ s^{-1}$	0.300	0.0	200	10.0	0.0	20.0	6.55×10^{-6}
$\delta R_2^{0,syn} = 10\ s^{-1}$	0.337	12.3	200	10.2	2.0	16.9	3.27×10^{-3}
$\delta R_2^{0,syn} = 15\ s^{-1}$	0.356	18.6	199	10.3	3.0	15.3	5.66×10^{-3}
$p_b^{syn} = 0.2$							
$\delta R_2^{0,syn} = 0\ s^{-1}$	0.200	0.0	200	10.0	0.0	20.0	5.98×10^{-6}
$\delta R_2^{0,syn} = 10\ s^{-1}$	0.230	15.0	200	10.4	4.0	17.9	1.79×10^{-3}
$\delta R_2^{0,syn} = 15\ s^{-1}$	0.247	23.5	199	10.7	7.0	16.8	3.07×10^{-3}
$p_b^{syn} = 0.1$							
$\delta R_2^{0,syn} = 0\ s^{-1}$	0.100	0.0	200	10.0	0.0	20.0	5.31×10^{-6}
$\delta R_2^{0,syn} = 10\ s^{-1}$	0.116	16.0	200	10.5	5.0	18.9	7.82×10^{-4}
$\delta R_2^{0,syn} = 15\ s^{-1}$	0.126	12.6	200	10.8	8.0	18.4	1.31×10^{-3}

*Parameter optimizations were performed for R_2 dispersion data generated with the parameters $\tau_{ex}^{syn} = 10$ ms,$\delta\omega^{syn}/2\pi = 200$ Hz, and $R_2^{a0,syn} = 20\ s^{-1}$ and the p_b^{syn} and $\delta R_2^{0,syn}$ values listed in the left most column in the Table. Parameters were optimized by minimizing Q^2 defined in Equation (1). Other symbols are defined in the footnotes in Table 1.

four-parameter fit assuming that $R_2^{0a} = R_2^{0b} = R_2^0$ would fit the slow exchange dispersion data as well as a five-parameter fit.

Parameter optimizations were also performed after introducing an rms uncertainty of 2% in all the synthetic R_2 data in order to compare the errors resulting from four-parameter fits with uncertainties introduced by random noise. The values of χ^2/N, listed in Table 4, for the Monte Carlo optimizations calculated for each synthetic data set are close to unity, indicating that the optimizations are consistent with the rms noise and small Q values listed in the Table 3.

As expected, Table 3 reveals that the optimized parameters p_b^{fit} and τ_{ex}^{fit}, derived from the four-parameter fits to the synthetic data, increasingly differ from their true values as δR_2^0 increases. These fitted parameters and $R_2^{0,fit}$ are plotted as functions of δR_2^0 in Figure 4. The curves in the figure depict the theoretical functional dependence of the fitted parameters on $\delta R_2^{0,syn}$, predicted by Equations 8–10. The plots show that there is excellent agreement between the predicted and fitted values of p_b and R_2^0. The theory also correctly predicts the observed linear increase in τ_{ex}^{fit} as $\delta R_2^{0,syn}$ increases as well as the increase in the error in τ_{ex}^{fit} as δp^{syn} decreases. However, the predicted errors are about twice those that are found numerically. In this one respect the theory performs less well in the case of slow exchange than it does for intermediate exchange (compare Figure 2(b) with 4(b)). This result is not unexpected because the accuracy of the Carver–Richards equation diminishes in the case of slow exchange (Tollinger et al., 2001). Finally, we note that errors introduced by the assumption that $R_2^{0a} = R_2^{0b}$ are far less than the uncertainties in p_b^{fit} and τ_{ex}^{fit} due to the 2% rms uncertainty in the synthetic R_2 data (Table 4). However, as we discuss below, by recording and fitting dispersion data for multiple sites and nuclei at several external fields, it is possible to greatly reduce experimental uncertainties in p_b^{fit} and τ_{ex}^{fit} obtained when fitting a single dispersion curve.

An assessment of impact the $R_2^{0a} = R_2^{0b}$ assumption on the accuracy of optimized exchange parameters

To estimate the parameter errors introduced by the $R_2^{0a} = R_2^{0b}$ assumption, we generated relaxation dispersion data assuming $R_2^{0a} \neq R_2^{0b}$ and then optimized the exchange parameters using the $R_2^{0a} = R_2^{0b}$ model. Interestingly, we got the excellent fit of the synthetic dispersion data using the $R_2^{0a} = R_2^{0b}$ model with a residual in R_2 less than 0.017%. This result also indicates that even when R_2^{0a} and R_2^{0b} are assumed as independent parameters to be optimized, the optimized values of R_2^{0a} and R_2^{0b} will have large uncertainties. Only when two independent peaks at a and b sites are

Table 4. Percentage uncertainties of the optimized exchange parameters listed in Table 1 due to a rms 2% uncertainty in the synthetic R_2 data

Initial parameters	Optimized values and percentage uncertainities				
	p_b^{fit}(%)	$\delta\omega^{fit}/2\pi$(%)	τ_{ex}^{fit}(%)	$R_2^{0,fit}$(%)	χ^2/N^*
$p_b^{syn} = 0.3$					
$\delta R_2^{0,syn} = 0\ s^{-1}$	26.9	5.6	28.0	0.58	0.99
$\delta R_2^{0,syn} = 10\ s^{-1}$	18.7	4.1	19.0	0.58	1.01
$\delta R_2^{0,syn} = 15\ s^{-1}$	14.6	3.6	15.0	0.49	1.03
$p_b^{syn} = 0.2$					
$\delta R_2^{0,syn} = 0\ s^{-1}$	42.3	5.4	43.0	0.59	0.99
$\delta R_2^{0,syn} = 10\ s^{-1}$	37.1	5.7	38.1	0.61	1.03
$\delta R_2^{0,syn} = 15\ s^{-1}$	25.9	4.3	26.1	0.59	1.01
$p_b^{syn} = 0.1$					
$\delta R_2^{0,syn} = 0\ s^{-1}$	91.0	8.3	100	0.56	1.01
$\delta R_2^{0,syn} = 10\ s^{-1}$	71.0	6.4	75.6	0.55	1.02
$\delta R_2^{0,syn} = 15\ s^{-1}$	75.1	7.3	77.6	0.55	0.98

*Uniform 2% rms uncertainties were assumed for the relaxation dispersion data. Uncertainities of the optimized values were estimated as the standard deviations of the parameter distributions derived from the Monte-Carlo calculations. The target function used for the minimization in each trial is given by χ^2 in Equation (2).

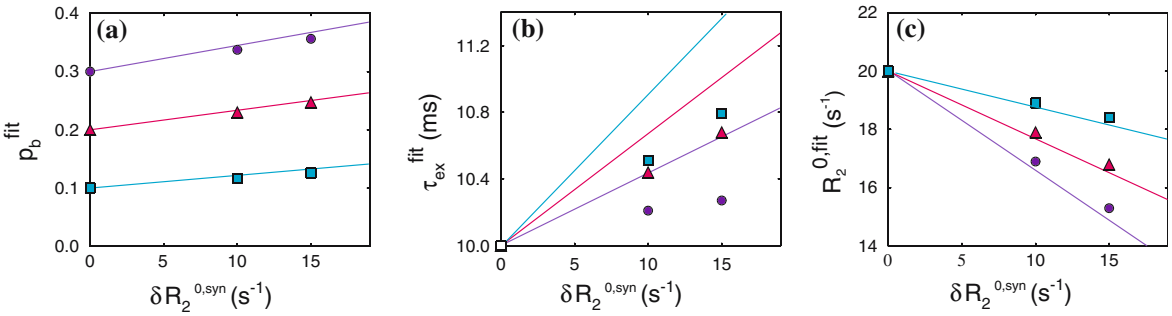

Figure 4. Plots of optimized exchange parameters (a) p_b^{fit}, (b) τ_{ex}^{fit}, and (c) $R_2^{0,fit}$ obtained by fitting slow-exchange R_2 data, generated assuming five parameters, with a four-parameter model. The synthetic data were generated using the following parameters: $p_b^{syn} = 0.1$, 0.2, and 0.3, $\tau_{ex}^{syn} = 10$ ms, $\delta\omega^{syn}/2\pi = 200$ Hz, $R_2^{0a,syn} = 20\ s^{-1}$, $\delta R_2^{0,syn} = 0$, 10, 15 s^{-1}. The optimized values at $p_b^{syn} = 0.1$, 0.2, and 0.3 are plotted using □ (cyan), Δ (red), and ○ (purple), respectively. The parameter values predicted by Equations (10), (9), and (8) for p_b^{fit}, τ_{ex}^{fit}, and $R_2^{0,fit}$, respectively, are plotted as three colored lines, corresponding to $p_b^{syn} = 0.1$ (cyan), 0.2 (red), and 0.3 (purple).

observed in slow exchange limit, R_2^{0a} and R_2^{0b} values may be determined using cross-correlated relaxation rate, η_{xy} (Wang et al., 2001). The use of η_{XY} is limited to the slow exchange limit because when $R_2^{0a} \neq R_2^{0b}$, η_{xy} is described as a cross-correlation rate with a weighted average of the rotational correlation times but not as a weighted average of η_{XY} values at site a and b. Therefore, in most cases except for the very slow exchange, it will be impossible to determine the true R_2^{0a} and R_2^{0b} values from the dispersion data. When there is a possibility of $R_2^{0a} \neq R_2^{0b}$ in the studying system, the R_2^0 value is a weighted averages of R_2^{0a} and R_2^{0b} and therefore it is not straight forward to use R_2^0 extract information about internuclear distances and correlation times.

The $R_2^{0a} = R_2^{0b}$ assumption did not introduce errors in $\delta\omega^{fit}$, but did introduce errors in p_b^{fit} and τ_{ex}^{fit}. In intermediate exchange, errors in p_b^{fit}, at $p_b^{syn} = 0.3$ and 0.2, (Table 1) were smaller than the uncertainties caused by the 2% rms random errors in R_2 (Table 2), whereas, errors in p_b^{fit} at $p_b^{syn} = 0.1$ (Table 1) were larger than the uncertainties caused by the random errors. Errors in τ_{ex}^{fit} in intermediate exchange (Table 1) and errors in p_b^{fit} and τ_{ex}^{fit} in slow exchange (Table 3) were all less than the

uncertainties caused by the 2% rms random error in R_2 (Tables 2 and 4). In addition, the random errors in R_2 cause the uncertainties of the fitting parameters to increase, as the ratio $R_2(\nu_{CP} = 0)/R_2(\nu_{CP} \rightarrow \infty)$ decreases (i.e. as the fractional error in R_{ex} increases). Although these results indicate that the random error in the parameters is small, the accuracy of the optimized parameters can be increased by group fitting dispersion curves of multiple residues whose chemical shifts are modulated by a common conformational fluctuation (Beach et al., 2005; Ishima and Torchia, 2005). The accuracy of the optimized parameters can be further improved by fitting the R_2 dispersions of several types of spins at several temperatures and static magnetic fields (Grey et al., 2003; Choy et al., 2004). Therefore, when the group-fitting approach reduces parameter uncertainties to less than ca. 5%, the errors caused by the $R_2^{0a} = R_2^{0b}$ assumption should be estimated using Equations (7)–(10) and/or by obtaining estimates of parameter uncertainties using five parameter fitting.

The parameter errors introduced by the $R_2^{0a} = R_2^{0b}$ assumption propagate to the values of important thermodynamic quantities derived from the optimized parameters. Examples are the free-energy difference between a and b sites, $\Delta G = -\ln(p_b/p_a)$, and the dissociation constant, $K_D = (p_b/p_a)[L]$, where [L] is the ligand concentration. Equation (10) predicts that the fractional error of (p_b^*/p_a^*) introduced by the $R_2^{0a} = R_2^{0b}$ assumption is $2\delta R_2^0 \tau_{ex}$ which is twice larger than the fractional error of τ_{ex}.

The errors in the optimized parameters caused by the $R_2^{0a} = R_2^{0b}$ assumption are accurately predicted by Equations (7)–(10) in the intermediate exchange regime where the Carver–Richard equation is highly accurate. In intermediate exchange, the theoretical equations correctly predict (Figure 2) that the fractional parameter errors increase as $\delta R_2^{0,syn}$ increases and as δp^{syn} increases. In slow exchange, the parameter errors are smaller than those predicted by the theoretical equations, because the Carver–Richard equation no longer predicts R_2 dispersion profiles with high accuracy (Tollinger et al., 2001) (Figure 4). In order to clarify how the optimized τ_{ex}^{fit} values compare with theoretical predictions, the optimized τ_{ex}^{fit} values are plotted against p_b^{syn} in Figure 5 in for p_b^{syn} in the range from 0.02 to 0.3. In the intermediate exchange case (Figure 5a) τ_{ex}^{fit} linearly increases as

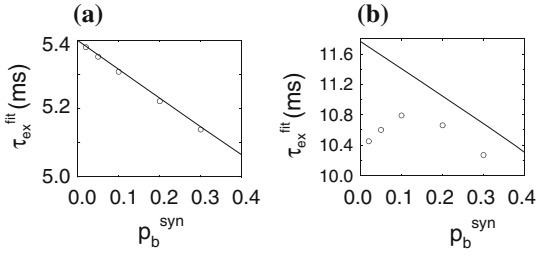

Figure. 5 Plots of τ_{ex}^{fit} values (○) against p_b^{syn} obtained using the $R_2^{0a} = R_2^{0b}$ model to fit (a) intermediate and (b) slow exchange R_2 dispersion data. The parameters used in (a) and (b) are those listed in Tables 1 and 3, respectively, with $\delta R_2^{0,syn} = 15\ s^{-1}$. The values of τ_{ex}^{fit} are compared with values predicted by Equation (9) and shown as solid lines.

p_b^{syn} decreases, in excellent agreement with the prediction of Equation (9). In contrast, in slow exchange (Figure 5b), Equation (9) overestimates τ_{ex}^{fit} (and the error in τ_{ex}^{fit}) at all values of p_b^{syn}, in particular as p_b^{syn} approaches zero. The latter observation is similar to previous reports (Millet et al., 2000; Korzhnev et al., 2004a) that, in slow exchange with $p_a >> p_b$, the errors in R_2 caused by the $R_2^{0a} = R_2^{0b}$ assumption are small. However, our simulations show that τ_{ex}^{fit} is not equal to τ_{ex}^{syn} even when p_b^{syn} approaches zero, indicating that in this limit the fit parameters are not free from errors. To understand this observation, we equated the slow-exchange $R_2(\nu_{cp} \rightarrow 0)$ and $R_2(\nu_{cp} \rightarrow \infty)$ equations in four parameters to the corresponding equations in five parameters (Equations (13) and (14), respectively), and thereby derived Equation (15).

$$R_2^{0*} + p_b^*/\tau_{ex}^* = R_2^a + p_b/\tau_{ex} \quad (\nu_{cp} \rightarrow 0) \qquad (13)$$

$$R_2^{0*} = p_a R_2^a + p_b R_2^b \quad (\nu_{cp} \rightarrow \infty) \qquad (14)$$

$$p_b^*/\tau_{ex}^* = (1 + \delta R_2^0 \tau_{ex}) p_b/\tau_{ex} \qquad (15)$$

We confirmed that the fit parameters in Figure 5b satisfied Equation (15). Nevertheless, in fitting experimental data, these errors will be undetectable because, as p_b approaches zero, R_{ex} becomes negligible for all values of ν_{CP}.

In spite of the fact that the $R_2^{0a} = R_2^{0b}$ assumption is often used in the analysis of the relaxation dispersion data, there are many practical cases in which $R_2^{0a} \neq R_2^{0b}$. Significant dif-

ferences between R_2^{0a} and R_2^{0b} would be expected when exchanging species are (1) unfolded and folded states of a protein (2) a protein monomer and oligomer (3) a ligand, free and bound to a large protein. When such obvious differences between R_2^{0a} and R_2^{0b} are expected, errors in the parameters caused by the $R_2^{0a} = R_2^{0b}$ assumption can be estimated using expected or simulated R_2^{0a} and R_2^{0b} values, as we discussed above. However, when the nature of the b-conformation is unknown, it will not be possible to estimate R_2^{0b}, and this will introduce uncertainties into the errors calculated for the optimized fitting parameters. Although our analysis has been restricted to two-site exchange, it is clear that similar errors will be introduced into parameters derived by fitting multi-site exchange data whenever $R_2^{0i} \neq R_2^{0j}$.

In summary, we note that because R_2 dispersion data can be well fit using an incorrect model (which assumes $R_2^{0a} = R_2^{0b}$) the optimized fitting parameters derived from such fits will typically have larger uncertainties than those calculated using random errors alone. Each synthetic dispersion data set contains 34 points. Because experimental profiles typically contain about a dozen data points the random errors in experiments will typically be larger than those listed in Tables 2 and 4. However, in practice many dispersion curves are simultaneously fit, which reduces the uncertainties in the optimized parameter due to random errors to the levels used in our calculations. Therefore we think that our calculations closely simulate practical cases. Although the errors introduced by the $R_2^{0a} = R_2^{0b}$ assumption are small, provided that $\delta R_2^0 \tau_{ex} << 1$, they can be greater than those due to random errors, when highly accurate optimized parameters are obtained by group-fitting numerous dispersion data sets. When fitted parameter uncertainties are small, the errors introduced by the $R_2^{0a} = R_2^{0b}$ assumption should be estimated either by using five parameter fitting, or, much more easily, by using Equations (7)–(10) whenever the Carver–Richards equation is valid.

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