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Assessment of the effects of increased relaxation dispersion data on the extraction of 3-site exchange parameters characterizing the unfolding of an SH3 domain

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Abstract

Recently a suite of six CPMG relaxation dispersion experiments has been described for quantifying millisecond time-scale exchange processes in proteins. The methodology has been applied to study the folding reaction of a G48M Fyn SH3 domain mutant that exchanges between the native state, and low populated unfolded and intermediate states. A complex non-linear global optimization protocol allows extraction of the kinetics and thermodynamics of the 3-site exchange process from the experimental data, as well as reconstruction of the amide group chemical shifts of the excited states. We show here, through a series of Monte-Carlo simulations on various synthetic data sets, that the 3-site exchange parameters extracted for this system on the basis of ^{15}N single-quantum (SQ) dispersion profiles exclusively, recorded at a single temperature, are significantly in error. While a temperature dependent ^{15}N study improves the robustness of extracted parameters, as does a combined analysis of ^{15}N and ^1H SQ data sets measured at a single temperature, the best agreement is observed in cases where the full complement of six dispersion profiles per residue is analyzed.

Relaxation dispersion NMR spectroscopy is a particularly versatile tool for the detection of excited conformational states that often are critical for biomolecular function (Palmer et al., 2001). In the past several years, interest has increased in using dispersion-based experiments for the investigation of biomolecular dynamics and concomitantly there have been significant methodological developments that have impacted on such studies (Palmer et al., 2001, 2005). In at least some of the cases reported the observed exchange dynamics are more complex than two-site (Tollinger et al., 2001; Grey et al., 2003; Tolkmachev et al., 2003; Korzhnev et al., 2004b) and this can complicate the extraction of robust parameters that define the exchange process.

With this in mind, our laboratory has recently used a suite of six CPMG-type experiments for studying exchange at backbone amide positions to characterize the folding reaction of an ^{15}N , ^2H labeled G48M mutant Fyn SH3 domain that exchanges between folded (F), low populated intermediate (I) and unfolded (U) states (Korzhnev et al., 2005). The experiments include ^1H and ^{15}N single-quantum (SQ) (Loria et al., 1999; Tollinger et al., 2001; Ishima and Torchia, 2003), ^1H - ^{15}N zero- (ZQ) and ^1H - ^{15}N double- (DQ) quantum (Orekhov et al., 2004), as well as ^1H and ^{15}N multiple-quantum (MQ) (Korzhnev et al., 2004a) dispersion measurements. A particularly important advantage of using the full suite of experiments is that there is a

considerable addition of data without the introduction of further adjustable exchange parameters, in comparison to ^1H and ^{15}N SQ data (Korzhnev et al., 2005). However, this advantage does come at a price, namely the need for ^2H labeling and the extra measurement time required to generate all six profiles, and it is of considerable interest, therefore, to compare the quality of fitted parameters in relation to the amount of data added. Here we show, based on analysis of synthetic data generated using exchange parameters that are obtained for the G48M Fyn SH3 system, that there are significant benefits to recording the full set of experiments relative to only ^{15}N data sets.

Extraction of conformational exchange parameters from the dependence of the experimental $R_{2,\text{eff}}^{\text{exp}}$ transverse relaxation rates on the CPMG frequency, ν_{CPMG} , is usually performed by least-square fits of calculated $R_{2,\text{eff}}^{\text{clc}}$ rates to experimental values by minimization of a χ^2 target function:

$$\chi^2(\zeta) = \sum \frac{\left(R_{2,\text{eff}}^{\text{clc}}(\zeta) - R_{2,\text{eff}}^{\text{exp}}\right)^2}{\left(\Delta R_{2,\text{eff}}^{\text{exp}}\right)^2}, \quad (1)$$

where $\Delta R_{2,\text{eff}}^{\text{exp}}$ are the uncertainties in the experimental $R_{2,\text{eff}}^{\text{exp}}$ rates, $\zeta = \{x_1, \dots, x_{\text{npar}}\}$ denotes the set of adjustable model parameters and the summation in Eq. 1 is over all experimental data points, including, where available, all six dispersions mentioned above. The $R_{2,\text{eff}}^{\text{clc}}$ rates depend non-linearly on the exchange rate constants $k_{\text{ex},ij}$ (sum of forward, k_{ij} , and reverse, k_{ji} , rate constants for the exchange between states i and j), on the populations of the exchanging states p_i , on the ^1H (^{15}N) chemical shift differences $\Delta\omega_{\text{H},ij}$ ($\Delta\omega_{\text{N},ij}$) expressed in ppm (or, equivalently, $\Delta\omega_{\text{H},ij}$ ($\Delta\omega_{\text{N},ij}$) in rad/s), and on the intrinsic R_2 rates for the coherences that evolve during the CPMG relaxation intervals in each of the experiments. The exact functional form describing the dependence of $R_{2,\text{eff}}^{\text{clc}}$ on the parameters of 2- and 3-site exchange models are presented elsewhere (Korzhnev et al., 2005). The summation in Eq. 1 includes data for all amide groups involved in the same exchange process. In this case the exchange rate constants $k_{\text{ex},ij}$ and populations p_i are *global* parameters, common for all residues included in the sum, whereas $\Delta\omega_{\text{H},ij}$, $\Delta\omega_{\text{N},ij}$ and the intrinsic R_2 rates are specific for each amide group.

In the case of a 2-site exchange process ($F \leftrightarrow U$), fitting of SQ (^1H or ^{15}N) dispersion data for

individual residues measured at multiple magnetic fields most often results in a unique solution for $k_{\text{ex},\text{FU}} = k_{\text{FU}} + k_{\text{UF}}$, p_U and $|\Delta\omega_{\text{FU}}|$, although in some limiting cases it is not possible to extract all exchange parameters separately (i.e., in the limit of slow exchange, $k_{\text{ex},\text{FU}} \ll |\Delta\omega_{\text{FU}}|$, the product $k_{\text{ex},\text{FU}}p_U$ can often only be obtained, while if $k_{\text{ex},\text{FU}} \gg |\Delta\omega_{\text{FU}}|$, $k_{\text{ex},\text{FU}}$ and $p_U(1 - p_U)\Delta\omega_{\text{FU}}^2$ can only be extracted). A combined analysis of ^1H and ^{15}N SQ, ^1H - ^{15}N ZQ, ^1H - ^{15}N DQ, ^1H and ^{15}N MQ dispersion data performed on a per-residue basis using the 2-site exchange model also results in a well defined solution for $k_{\text{ex},\text{FU}}$, p_U , $\Delta\omega_{\text{N},\text{FU}}$ and $\Delta\omega_{\text{H},\text{FU}}$, although only the relative signs of $\Delta\omega_{\text{N},\text{FU}}$ and $\Delta\omega_{\text{H},\text{FU}}$ are obtained directly from the dispersion data.

In contrast, finding the optimal solution in the case of a system described by a 3-site exchange process ($F \leftrightarrow I \leftrightarrow U$) is much more complicated (Korzhnev et al., 2005). First, in some exchange regimes, the 3-site exchange process can be effectively described by a 2-site mechanism, making it difficult to extract reliable 3-site exchange parameters and to discriminate between 2- and 3-site models. For example, if the reaction $I \leftrightarrow U$ is fast, the $F \xrightleftharpoons[k_{\text{IF}}]{k_{\text{FI}}} I \xrightleftharpoons[k_{\text{UI}}]{k_{\text{IU}}} U$ reaction essentially reduces to $F \leftrightarrow U/I$ with $(p_{\text{F}})^{2\text{-site}} = p_{\text{F}}$, $(p_{\text{U/I}})^{2\text{-site}} = p_{\text{I}} + p_{\text{U}}$, $(k_{\text{ex}})^{2\text{-site}} = k_{\text{FI}}/(p_{\text{I}} + p_{\text{U}})$, and $(\Delta\omega)^{2\text{-site}} = (p_{\text{I}}\Delta\omega_{\text{FI}} + p_{\text{U}}\Delta\omega_{\text{FU}})/(p_{\text{I}} + p_{\text{U}})$, so that all combinations of 3-site exchange parameters that satisfy the above relations result in similar χ^2 values. In fact, as described previously (Korzhnev et al., 2005), the uncertainties in experimental $R_{2,\text{eff}}^{\text{exp}}$ values (typically 1–4%) are usually too high for the effective discrimination between 2- and 3-site exchange on the basis of any kind of individual dispersion profile measured for a single amide group (for example, based exclusively on ^{15}N or ^1H SQ data). Thus, selection between 2- and 3-site models requires a combined analysis of relaxation dispersion data for multiple residues and/or different types of relaxation dispersion profiles. Second, χ^2 surfaces even for individual amide groups, may exhibit multiple non-equivalent minima beyond the pair of equivalent minima that arise due to a degeneracy with respect to sign inversion of chemical shift differences (for a 3-site exchanging system solutions with chemical shift differences of $(\Delta\omega_{\text{FI}}, \Delta\omega_{\text{FU}})$ and $(-\Delta\omega_{\text{FI}}, -\Delta\omega_{\text{FU}})$ are degenerate) (Korzhnev et al., 2004b). Consider, for example, the χ^2 error surfaces, Figure 1, generated from fits of simulated, noise-free

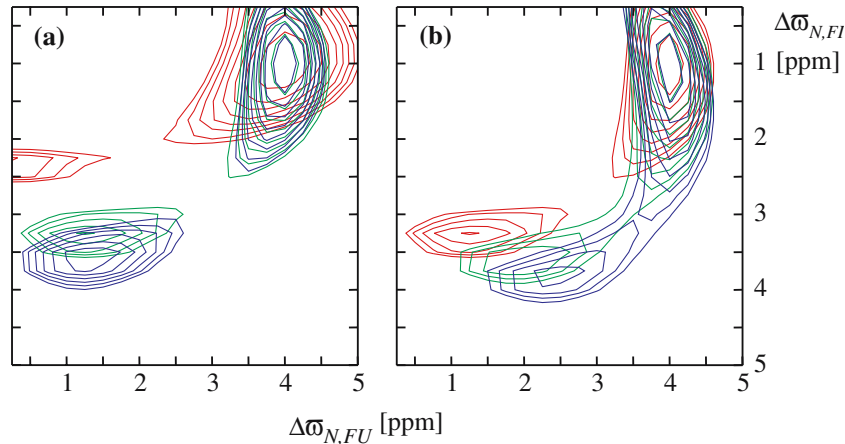


Figure 1. Spurious minima on the $\chi^2(\Delta\varpi_{N,FI}, \Delta\varpi_{N,FU})$ surface obtained from fitting simulated noise-free ^{15}N SQ CPMG relaxation dispersion data for a single nucleus using a 3-site exchange model (40 degrees of freedom). Synthetic noise-free ^{15}N SQ data, $R_{2,\text{eff}}$, were generated (CPMG frequencies ranging from 50 to 1000 Hz), for static magnetic fields of 14.1 T and 18.8 T with $\Delta\varpi_{N,FI} = 1.0$ ppm; $\Delta\varpi_{N,FU} = 4.0$ ppm; $k_{\text{ex},FI} = 1000/\text{s}$; $k_{\text{ex},IU} = 1000/\text{s}$ (red in a), 3000/s (green in a, all simulations in b), 5000/s (blue in a); $p_I = 2\%$; $p_U = 2\%$ (a, red in b), 3% (green in b), 4% (blue in b) and intrinsic transverse relaxation rates R_2 of 12 s^{-1} (14.1 T) and 14 s^{-1} (18.8 T). For the calculation of χ^2 values, uncertainties of 2% were assumed for $R_{2,\text{eff}}$. χ^2 contours are plotted in steps of 25 with $\chi^2 = 0$ at the correct minimum. Note the second false minimum that appears at different positions depending on the values of the exchange parameters and shift differences.

^{15}N SQ dispersion data for a single probe using exchange parameters listed in the legend to the Figure. The surfaces were generated as a function of $\Delta\varpi_{N,FI}$ and $\Delta\varpi_{N,FU}$, assuming the correct values of $k_{\text{ex},FI}$, $k_{\text{ex},IU}$, p_I and p_U . It is clear, that in addition to the χ^2 minimum at the correct values of $\Delta\varpi_{N,FI}$ and $\Delta\varpi_{N,FU}$ ($\Delta\varpi_{N,FI} < \Delta\varpi_{N,FU}$), there is an artificial minimum with $\Delta\varpi_{N,FI} > \Delta\varpi_{N,FU}$, (Figure 1a,b) whose depth and position depend on the exchange parameters. Although the difference in χ^2 between these minima is often sufficiently large for discrimination if the correct combination of $k_{\text{ex},FI}$, $k_{\text{ex},IU}$, p_I and p_U is used, this is often no longer the case on the higher-dimensional surface $\chi^2(k_{\text{ex},FI}, k_{\text{ex},IU}, p_I, p_U, \Delta\varpi_{N,FI}, \Delta\varpi_{N,FU})$. Global minimization of ^{15}N dispersion data involving multiple sites helps alleviate this problem by narrowing down the range of exchange parameters $k_{\text{ex},FI}$, $k_{\text{ex},IU}$, p_I and p_U . However, the number of local minima on the global χ^2 surface (the sum of χ^2 for individual residues) is substantial and, at least in studies of the Fyn SH3 domain, additional data is needed (Korzhnev et al., 2004b).

There are several ways to eliminate (or at least to significantly reduce the number of) spurious minima on the χ^2 surface. As described above use of the suite of six dispersion profiles results in

additional data without the introduction of further adjustable exchange parameters, eliminating many of the artificial χ^2 minima. In order to evaluate the influence of additional data sets on the extracted parameters, we have performed a series of Monte Carlo simulations (Press et al., 1988) of a 3-site exchange reaction, using exchange parameters listed in the first row of Table 1. A suite of 6 synthetic dispersion profiles (^1H SQ, ^{15}N SQ, ^1H MQ, ^{15}N MQ, $^1\text{H}-^{15}\text{N}$ ZQ, $^1\text{H}-^{15}\text{N}$ DQ) were generated for 47 residues using values of $\Delta\varpi_{\text{H},FI}$, $\Delta\varpi_{\text{H},FU}$, $\Delta\varpi_{N,FI}$, $\Delta\varpi_{N,FU}$ and intrinsic relaxation rates that were obtained previously from an analysis of the experimental $^1\text{H}-^{15}\text{N}$ dispersion data measured on a perdeuterated G48M Fyn SH3 domain at 25 °C (Korzhnev et al., 2005). Sets of profiles were simulated for magnetic fields of 11.7, 14.4 and 18.8 T. Normally distributed random noise with a standard deviation of 2% (^1H SQ, ^{15}N SQ, ^1H MQ, and ^{15}N MQ data) or 4% ($^1\text{H}-^{15}\text{N}$ ZQ and DQ data) was subsequently added to each data set and a series of 20 Monte-Carlo simulations was performed. The ‘global’ χ^2 optimizations were performed using a protocol that has been described in detail previously involving sampling of many different initial conditions to select the deepest (global) χ^2 minimum (Korzhnev et al., 2005). All

Table 1. Parameters of the 3-site exchange folding reaction, $F \leftrightarrow I \leftrightarrow U$, used to generate synthetic dispersion data

Dataset	T [°C]	$k_{\text{ex,FI}}$ [s^{-1}]	$k_{\text{ex,IU}}$ [s^{-1}]	p_I [%]	p_U [%]
^a Deuterated G48M Fyn SH3 Suite of 6 dispersion profiles	25	4673	6756	0.73	5.13
^b Partially deuterated G48M Fyn SH3	20	2318	6957	0.56	2.61
	25	3184	9268	0.70	3.52
¹⁵ N SQ data	30	4331	12243	0.86	4.69

^aThe rates and populations were calculated from fits to experimental data recorded at 25 °C. A suite of 6 dispersion profiles were obtained/residue.

^bThe rates and populations were calculated from fits of experimental ¹⁵N SQ profiles over 3 temperatures, assuming that rates obey transition state theory and that chemical shift differences are temperature invariant. It is noteworthy that the exchange parameters are different for the two sets of samples, due to the differences in levels of deuteration.

47 residues were used in fits involving both ¹H and ¹⁵N dispersion data; 7 of the 47 residues were excluded from analysis when ¹⁵N SQ data exclusively was considered since the dispersions for these sites were too small ($<3 \text{ s}^{-1}$).

Distributions of exchange parameters $k_{\text{ex,FI}}$, $k_{\text{ex,IU}}$, p_I and p_U , along with the mean and standard deviation of each distribution, obtained from the Monte-Carlo analyses of ¹⁵N SQ data exclusively, a combination of ¹H and ¹⁵N SQ data and the suite of 6 dispersions are illustrated in Figure 2 (columns a–c respectively). It is clear that the values are poorly reproduced when ¹⁵N SQ relaxation dispersion profiles are considered exclusively (a), with exchange rates of several outliers off by as much as a factor of 4. The inclusion of ¹H SQ data (b) eliminates these outliers and improves the precision and accuracy of the extracted parameters significantly; it is noteworthy that, in addition to the statistical advantage of adding more data, the inclusion of ¹H dispersion profiles specifically allows better sampling of fast exchange processes relative to ¹⁵N CPMG experiments, since higher ¹H pulse repetition rates can be used (Ishima and Torchia, 2003). A still more narrow distribution of values is obtained when data from all six dispersions is included (c). It is interesting to note that the distributions in the extracted parameters obtained from the Monte-Carlo analysis are in good agreement with the parameter errors estimated on the basis of covariance matrix approaches (Press et al., 1988). For a non-linear model, random experimental noise with zero mean does not necessarily translate into the correct expectation value for the extracted parameters, which may explain the tendency

towards slightly smaller exchange rates $k_{\text{ex,FI}}$ offset by slightly larger intermediate state populations p_I in the simulations.

Figure 3a–c, shows a comparison of chemical shift differences extracted from Monte-Carlo simulations relative to the reference (input) shift differences. Although global fits of ¹⁵N dispersion data exclusively produce accurate values of $\Delta\varpi_{\text{N,FU}}$, it is clear that $\Delta\varpi_{\text{N,FI}}$ values are much less well defined, reflecting the differences in the populations of the I and U states (0.7 and 5%, respectively). In contrast, when all 6 dispersion profiles are used in concert (c), $\Delta\varpi_{\text{N,FI}}$ and $\Delta\varpi_{\text{H,FI}}$ can be determined to approximately 0.5 ppm and 0.1 ppm, respectively, for the vast majority of residues. In the case of V55 it is noteworthy that $\Delta\varpi_{\text{N,FU}} = -16.5$ ppm, and the large shift difference likely complicates the extraction of accurate parameters from CPMG data.

In cases where a perdeuterated sample is not available for recording the suite of 6 dispersion curves (see Ishima and Torchia (2003) for a discussion of the importance of deuteration for measuring CPMG profiles with ¹H pulsing) there is an alternative approach for elimination of artificial minima that involves the use of data recorded at multiple temperatures (Korzhnev et al., 2004b). The idea behind this method is illustrated simply in Figure 1a,b, where the position of minima in the $\chi^2(\Delta\varpi_{\text{N,FI}}, \Delta\varpi_{\text{N,FU}})$ surface obtained from fits of simulated noise-free ¹⁵N SQ data are shown, with different colored contours corresponding to χ^2 surfaces that are generated with different rates and/or populations but with the same chemical shift differences. This ‘simulates’ a temperature dependent series where the rates/populations would be expected to

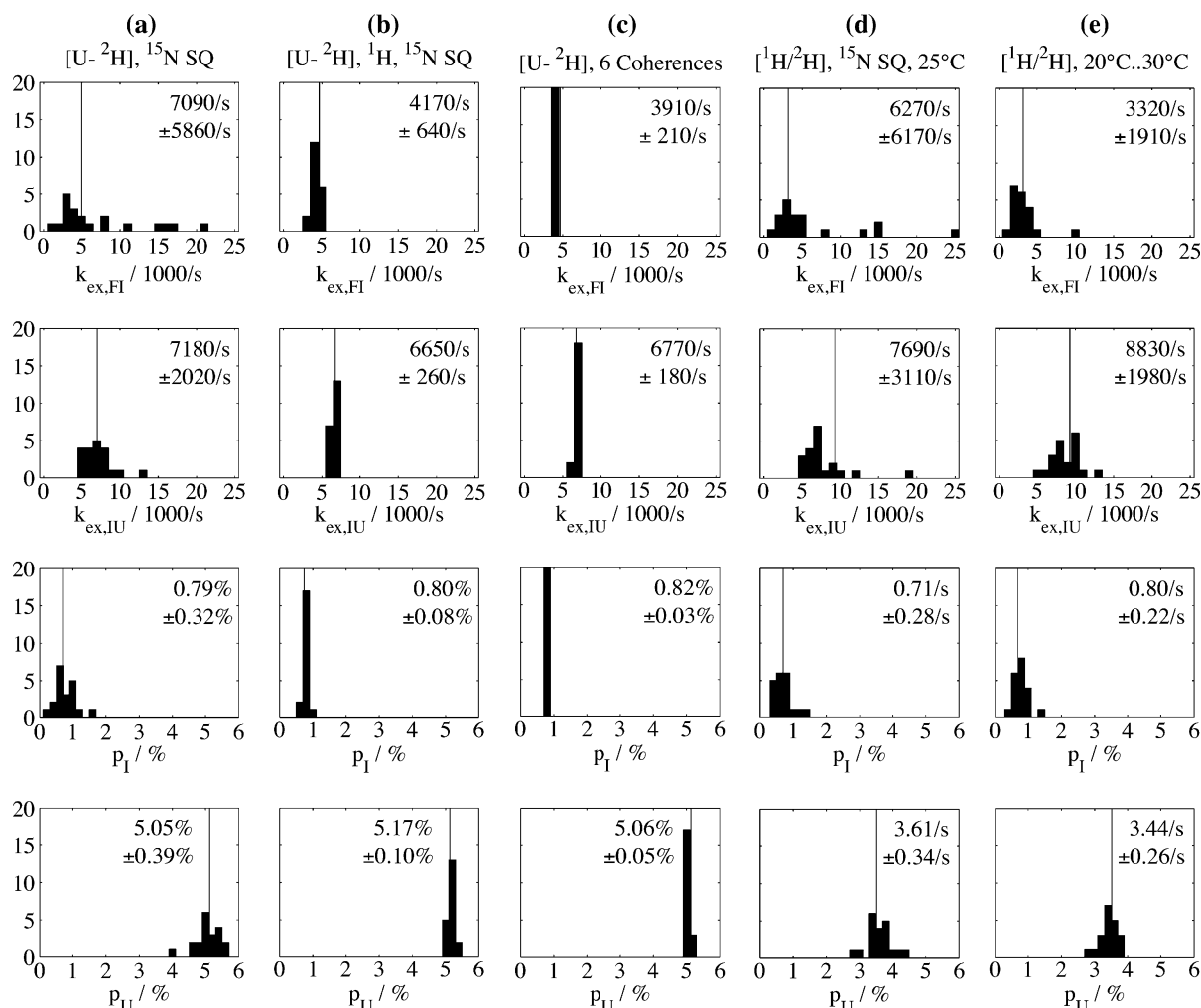


Figure 2. Histograms showing the distributions of 3-site exchange parameters $k_{\text{ex,FI}}$, $k_{\text{ex,IU}}$, p_I and p_U (from top to bottom) obtained from lowest ‘global’ χ^2 fits of Monte-Carlo simulations involving synthetic ¹⁵N SQ dispersion data (column a), synthetic ¹⁵N and ¹H SQ data (column b) and a suite of 6 dispersion profiles (column c) generated with exchange parameters from the first line of Table 1 that are obtained from experiments recorded on a [U-²H] sample (one temperature). Also shown are exchange parameters from fits (with assumptions as described in the text) of simulated dispersion data at a single (d) or multiple (e) temperatures that were generated from exchange parameters obtained from fits of experimental data recorded on a fractionally deuterated sample, [¹H/²H] and listed under ‘Partially deuterated G48M Fyn SH3 domain’ in Table 1. The target values (Table 1) are indicated by vertical lines. Also shown are the averages and standard deviations for each of the distributions.

change with no (little) change in shifts. The correct minimum in $(\Delta\varpi_{\text{N,FI}}, \Delta\varpi_{\text{N,FU}})$ space is invariant to the input rates, while the position of the false minimum depends in some complex way on both rates and shifts and thus moves with ‘temperature’. In this way χ^2 surfaces generated from fits of dispersion data recorded at several temperatures (under the assumption that chemical shifts are temperature invariant) will

contain fewer minima than the corresponding surfaces associated with single temperature data, leading to more robust estimates of exchange parameters. It is also possible to simplify the error surface by enforcing that the exchange rates follow simple transition state theory (an assumption), as described previously (Korzhev et al., 2004b). This is particularly useful in cases where at some, but not all, of the temperatures

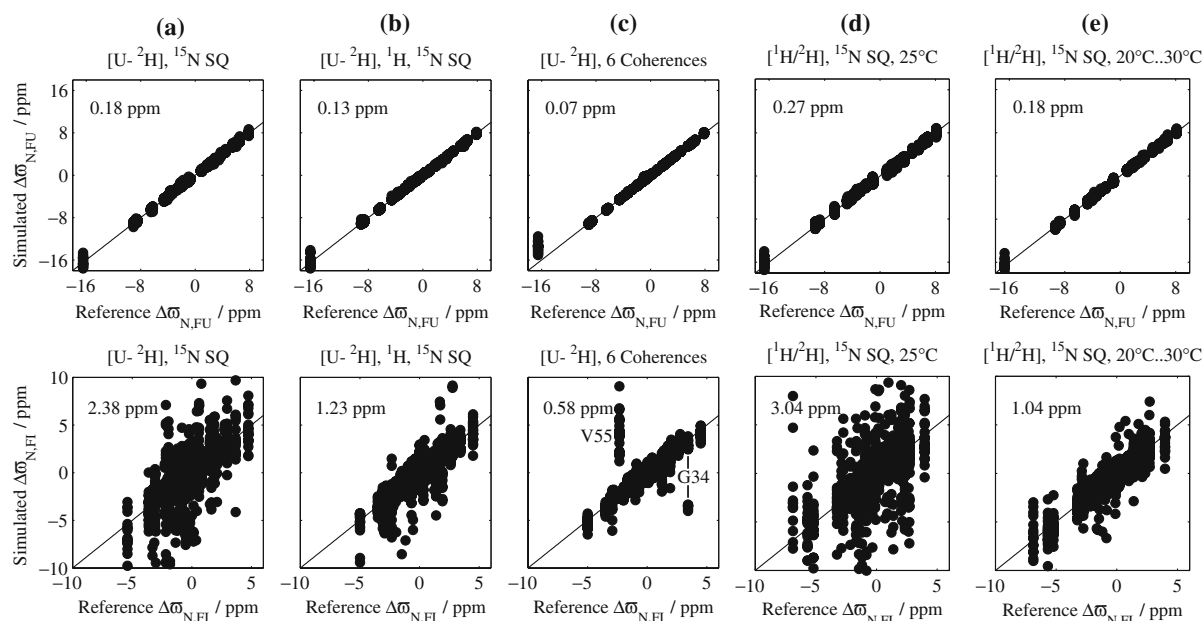


Figure 3. Distributions of the chemical shift differences $\Delta\sigma_{N,FU}$ and $\Delta\sigma_{N,FI}$ from a set of 20 Monte-Carlo simulations as in Figure 2, with columns a–c corresponding to data sets generated using input exchange parameters from line 1 of Table 1, and columns d,e based on simulations involving input parameters from lines 2–4 of the Table. Solid lines corresponding to $y=x$ are indicated. The RMSDs from the input values are specified in the upper left-hand corner of each panel. Shifts of V55 and G34 were excluded from the calculation of the RMSD in column c.

the 3-site reaction reduces effectively to a 2-site exchange process, such as would be the case if one of the exchange events becomes fast.

In order to evaluate how the addition of temperature dependent data influences the parameters extracted from fits involving ^{15}N SQ dispersion profiles exclusively we have carried out an additional series of 20 Monte-Carlo simulations. Here data sets were generated using reference parameters that had been obtained from a temperature dependent study involving a fractionally deuterated, ^{15}N sample of the G48M Fyn SH3 domain (Mittermaier et al., 2005) for which ^{15}N SQ relaxation dispersion profiles were obtained at temperatures of 20 °C, 25 °C, and 30 °C and magnetic field strengths of 500 MHz (20 °C and 30 °C only), 600 MHz (25 °C only), and 800 MHz (all three temperatures). The parameters used in the simulation were extracted from experimental data assuming that chemical shift differences between states are temperature independent and that rates obey transition state theory; they are listed in Table 1 (under “Partially deuterated G48M Fyn SH3”). Simulations were also carried out for the ^{15}N data recorded at 25 °C only so that

the improvement from inclusion of profiles recorded at the additional temperatures could be evaluated properly.

When simulated ^{15}N dispersion data at a single temperature only (25 °C) were fit (40 residues), extracted exchange parameters are not well defined, Figure 2 (column d). The addition of data at 20 and 30 °C improves the robustness of the fits significantly, with the distribution of extracted exchange parameters for 25 °C shown in Figure 2, column e. Here all temperature dependent synthetic data were analyzed assuming temperature invariance of $\Delta\sigma_{N,FU}$, $\Delta\sigma_{N,FI}$ along with kinetics described by transition state theory. Column d of Figure 3 compares $\Delta\sigma_{N,FU}$ and $\Delta\sigma_{N,FI}$ values extracted from the fits at 25 °C with the target values and it is clear that while $\Delta\sigma_{N,FU}$ values are well defined, $\Delta\sigma_{N,FI}$ values are very poorly reproduced. As with the exchange parameters, inclusion of additional temperatures improves the accuracy of the extracted shift differences, $\Delta\sigma_{N,FI}$, Figure 3, column e.

In summary, we have presented simulations, based on parameters describing the folding reaction of a G48M Fyn SH3 domain, showing that

the analysis of ^{15}N dispersion data recorded exclusively at a single temperature is not sufficient to extract accurate kinetic data for a 3-site exchanging system. Although inclusion of additional temperature data (along with a number of assumptions regarding the temperature dependence of the data, see above) does improve the robustness of the fits, the best agreement with input parameters is obtained when data recording all 6 dispersion types is fitted (see Figure 2). The availability of all dispersion experiments allows one to exploit the information encoded in the ZQ/DQ profiles regarding the relative signs of ^{15}N and ^1H chemical shift differences that is critical for the reconstruction of the chemical shifts of the excited states. The amount of data that is necessary for the robust extraction of parameters will depend to some extent on the exchange parameters themselves, on the number of amide groups that are involved in the exchange process, on the distributions of chemical shift differences, as well as on the experimental errors. One practical approach might be to first obtain and analyze ^{15}N and ^1H SQ dispersion profiles. An error analysis of the extracted rates, populations and shifts of the sort described here, would then establish whether additional data is necessary to minimize uncertainties in parameters, in which case further profiles could be recorded. The present set of simulations examines only a limited set of exchange conditions. Nevertheless, our results do underscore the importance of recording dispersion data sets that make use of a variety of coherences, for the robust extraction of the full complement of information that is available from CPMG dispersion experiments that quantify multi-site (>2) exchange processes.

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