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ARTICLE

Simple tests for the validation of multiple field spin relaxation data

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Abstract ^{15}N spin relaxation data is widely used to extract detailed dynamic information regarding bond vectors such as the amide N–H bond of the protein backbone. Analysis is typically carried using the Lipari–Szabo model-free approach. Even though the original model-free equation can be determined from single field R_1, R_2 and *NOE*, over-determination of more complex motional models is dependent on the recording of multiple field datasets. This is especially important for the characterization of conformational exchange which affects R_2 in a field dependent manner. However, severe artifacts can be introduced if inconsistencies arise between experimental setups with different magnets (or samples). Here, we propose the use of simple tests as validation tools for the assessment of consistency between different datasets recorded at multiple magnetic fields. Synthetic data are used to show the effects of inconsistencies on the proposed tests. Moreover, an analysis of data currently deposited in the BMRB is performed. Finally, two cases from our laboratory are presented. These tests are implemented in the open-source program *relax*, and we propose their use as a routine check-up for assessment of multiple field dataset consistency prior to any analysis such as model-free calculations. We believe this will aid in the extraction of higher quality dynamics information from ^{15}N spin relaxation data.

Keywords Consistency test · Model-free analysis · Spin relaxation · Protein dynamics · *relax* program

Introduction

NMR is a very powerful tool for probing protein dynamics at atomic resolution. The most often used approach to protein backbone dynamics is the recording of ^{15}N spin relaxation data to probe amide vectors motions. Recorded ^{15}N-R_1, ^{15}N-R_2 and $\{^1H\}^{15}$N-*NOE* are then analyzed using the Lipari–Szabo model-free approach (Lipari and Szabo 1982a, b). To over-determine extended model-free equations (Clore et al. 1990; d'Auvergne and Gooley 2008a), it is necessary to record the rates at multiple magnetic fields. However, combining multiple-field datasets is not trivial and, in some circumstances, can lead to artifacts. Indeed, this problem has previously been discussed in the literature (Fushman et al. 1998, 1999; Lee and Wand 1999).

Indeed, to extract high quality information from multiple field experiments, it is important that datasets share a high degree of consistency, as combining inconsistent datasets is similar to combining datasets recorded under different experimental conditions, i.e. potentially reporting on different motions. Inconsistencies can arise from several factors, including variations in sample viscosity (caused by changes in temperature and concentration), water saturation during acquisition (which influences N–H moieties as a function of the exchange rate with the aqueous solvent), other differences in data acquisition between different NMR spectrometers or NMR centers, and any form of time-dependent sample modifications (binding state, degradation

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and aggregation). If variations are present among the datasets recorded at different magnetic fields, then recorded spin relaxation parameters will be inconsistent with each other.

The effects of combining inconsistent datasets can vary depending on the source of the problem. In certain cases, incorrect characterization of global tumbling can arise, indirectly leading to erroneous evaluation of local dynamics (Schurr et al. 1994; Tjandra et al. 1996b). Moreover, artifactual μs–ms motions can be falsely detected as non-null R_{ex} parameters Finally, inconsistent datasets can yield to some N–H vectors being rejected from the analytical scheme because of too high χ^2 statistics, thus reducing the information content obtained from the experiments. No matter the exact situation, however, it is clear that inconsistent datasets will lead to mistaken descriptions of local motions and should be avoided.

In this study, three field independent functions are proposed to assess the consistency of multiple magnetic field transversal relaxation rates within spin relaxation datasets aimed at model-free analysis. The goal of these tests is similar to the self-consistency test previously proposed by Fushman et al. (1999). The use of the three tests we now propose, for which we show the efficiency using both synthetic and experimental data, should become routine so dataset quality is assessed prior to derivation of dynamics parameters. This additional step in ^{15}N spin relaxation studies is believed to allow more relevant data to be extracted from the information rich R_1, R_2 and *NOE*, avoiding experimental errors such as those arising from temperature or concentration deviations.

Methods

Consistency tests

Three consistency tests can be used in the proposed approach: $J(0)$ (Farrow et al. 1995), F_η and F_{R_2} (Fushman et al. 1998). These field independent functions using R_1, R_2 and *NOE* as input parameters (which we implemented in the program *relax*, versions 1.2.14 and higher) (d'Auvergne and Gooley 2008a, b) consist of:

$$J(0) = \frac{-1.5}{3d + c}\left(\frac{R_1}{2} - R_2 + 0.6\sigma_{\mathrm{NOE}}\right) \quad (1)$$

$$F_\eta = \frac{\eta}{B_0\{4 + 3[1 + (\omega_{\mathrm{N}}\,\tau_m)^2]^{-1}\}} \quad (2)$$

$$F_{R_2} = \frac{R_2 - P_{\mathrm{HF}}}{\{4 + 3[1 + (\omega_{\mathrm{N}}\tau_m)^2]^{-1}\}(d + c/3)} \quad (3)$$

where $d = (1/4)(\mu_0/4\pi)^2(\hbar\gamma_{\mathrm{N}}\gamma_{\mathrm{H}})^2/r_{\mathrm{N-H}}^6$ is the dipolar constant, $c = (\omega_{\mathrm{N}}\,\Delta\sigma)^2/3$ is the chemical shift anisotropy (*CSA*) constant, μ_0 is the permeability of free space, $\hbar$ is Planck's constant divided by 2π, γ_{N} and γ_{H} are the gyromagnetic ratios of ^{15}N and ^{1}H, respectively, $\omega_{\mathrm{N}} = B_0\gamma_{\mathrm{N}}$ is the Larmor frequency of ^{15}N, B_0 is the static magnetic field strength, $r_{\mathrm{N-H}}$ is the vibrationally averaged effective N–H bond length, $\Delta\sigma = \sigma_{\parallel} - \sigma_{\perp}$ is the *CSA*, $\sigma_{\mathrm{NOE}} = (NOE - 1)R_1\gamma_{\mathrm{N}}/\gamma_{\mathrm{H}}$, $\eta = 0.5\sqrt{dc/3}[4J(0) + 3J(\omega_{\mathrm{N}})][3(\cos\theta)^2 - 1]$ is the cross-correlation rate between ^{15}N *CSA* and ^{15}N–^{1}H dipolar interaction, $J(\omega_{\mathrm{N}}) = (R_1 - 1.4\sigma_{\mathrm{NOE}})/(3d + c)$ is the spectral density at the ^{15}N frequency, θ is the angle between the ^{15}N–^{1}H vector and the principal axis of the ^{15}N chemical shift tensor, τ_m is an estimated isotropic global correlation time for the system under study (expressed in ns), and $P_{\mathrm{HF}} = -1.3\sigma_{\mathrm{NOE}}$ is the contribution to R_2 from high frequency motions. For the calculations presented here, θ was 15.7° as in Fushman et al. (1998), $r_{\mathrm{N-H}}$ was 1.02 Å, and the ^{15}N *CSA* was assumed to be −172 ppm. Both $r_{\mathrm{N-H}}$ and ^{15}N *CSA* were typical of spin relaxation studies analysed using the model-free formalism.

Performing these simple calculations for each residue and then comparing results obtained at different magnetic fields should, in the case of perfect consistency and assuming the absence of conformational exchange, yield equal values independently of the magnetic field.

Synthetic datasets

Synthetic datasets were generated using the program *relax* (version 1.2.14) (d'Auvergne and Gooley 2008a, b). R_1, R_2 and *NOE* were calculated at three magnetic fields in the case of an N–H vector with an order parameter (S^2) of either 0.6, 0.7, 0.8, 0.9 or 1.0, and with a correlation time (τ_e) of either 20 or 100 ps (model *m*2 within the model-free analysis) on a molecule tumbling isotropically with a correlation time (τ_m) of either 5, 10, 20 or 40 ns.

Experimental datasets

Experimental datasets were retrieved from the BMRB (Ulrich et al. 2008). These were datasets recorded at two or three magnetic fields for proteins with apparent correlation times ≥5 ns (see Table 5 for a description of the method used to estimate τ_m). Moreover, two test cases for homologous proteins TEM-1 (Savard and Gagné 2006) and PSE-4 (Morin and Gagné 2009) were analysed in details to show the advantage of performing consistency tests.

Results

The magnetic field dependence of spin relaxation parameters (Abragam 1961, see Eqs. 4, 5 and 6 as well as Figs. S1–

S3 in the Supporting information) allows over-determination of model-free equations for quantification of both global and local dynamics within a molecular system. Recorded data is usually taken directly as input into minimisation procedures to yield model-free parameters. A field independent function based on these dynamics parameters would be useful to assess datasets consistency prior to these analyses. This would avoid the potential extraction of erroneous information as well as the waste of time associated to dissecting inconsistent datasets using numerous long model-free minimisations with different subsets of data.

$$R_1 = d[J(\omega_H - \omega_N) + 3J(\omega_N) + 6J(\omega_H + \omega_N)] + c\,J(\omega_N) \quad (4)$$

$$R_2 = \frac{d}{2}[4J(0) + J(\omega_H - \omega_N) + 3J(\omega_N) + 6J(\omega_H) + 6J(\omega_H + \omega_N)] + \frac{c}{6}[4J(0) + 3J(\omega_N)] + R_{ex} \quad (5)$$

$$NOE = 1 + \frac{\gamma_H}{\gamma_N}\frac{d}{R_1}[6J(\omega_H + \omega_N) - J(\omega_H - \omega_N)] \quad (6)$$

The three tests proposed in this study, $J(0)$, F_η and F_{R_2}, are presented in the "Methods" section. While $J(0)$ (Farrow et al. 1995), the spectral density at the zero frequency, is mainly used in studies of unfolded proteins using reduced spectral density mapping, it is very well suited for assessing consistency since it is, in the absence of μs–ms motions, a field independent function. On the other hand, F_η and F_{R_2} are two functions which were previously proposed for consistency testing in the case of determining the ^{15}N *CSA* using the cross-correlation rate η and the transversal relaxation rate R_2 (Fushman et al. 1998), and are also, in the absence of μs–ms motions, field independent functions. Performing these three tests for consistency testing purposes requires R_1, R_2 and *NOE* data at two magnetic fields or more. Moreover, an estimate of the global correlation time τ_m is required for tests F_η and F_{R_2}.

Consistency testing is performed by calculating the different functions using the experimental data from each magnetic field and then comparing values obtained on a per residue basis before having a look at the distribution of ratios. This global picture indicates the level of consistency in a qualitative manner, where consistent datasets have distributions of ratios centered at one, while deviations from one are observed for inconsistent datasets. Examples of consistency analyses are shown in Figs. 1 and 2.

Consistency tests of synthetic datasets

Simulations based on synthetic data confirm the field independent nature of the $J(0)$, F_η and F_{R_2} functions in the case of consistent datasets for spins not affected by slow μs–ms motions (Table 1, see also Figs. S4–S6 in the

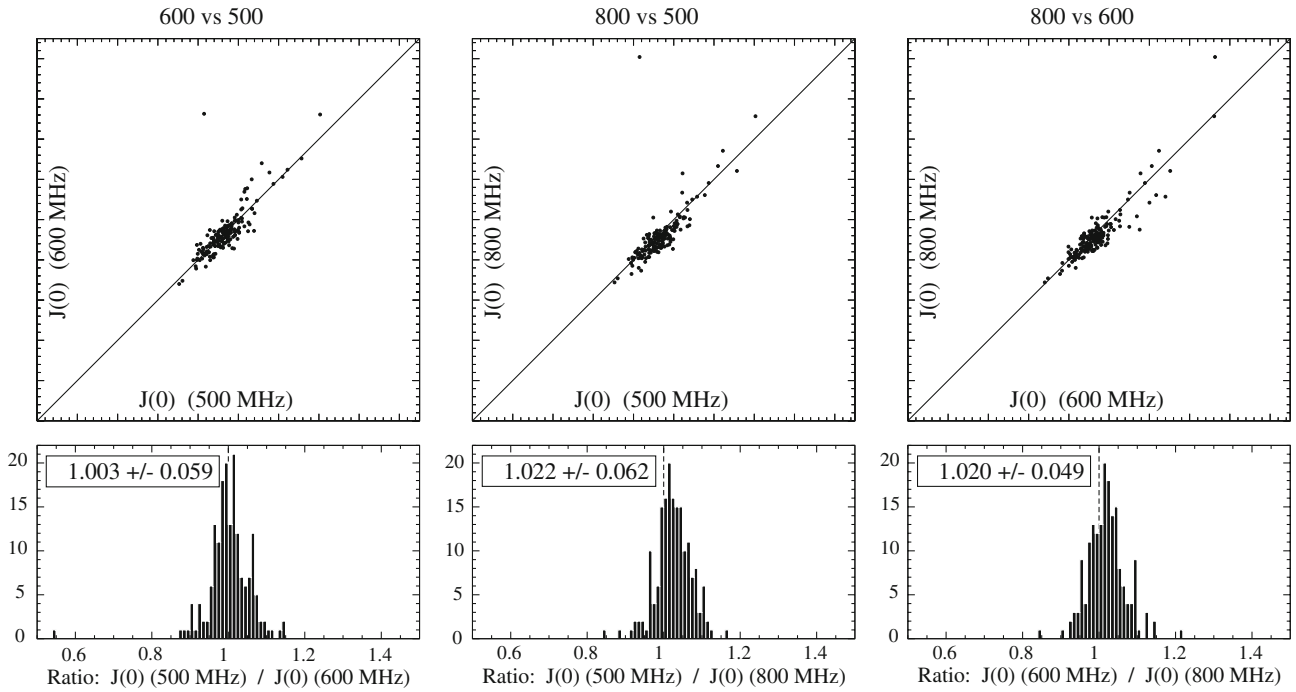

Fig. 1 $J(0)$ consistency of datasets for TEM-1. The consistency of these three magnetic field datasets is high. Since no *NOE*s were recorded at 800 MHz, the $J(0)$ values at 800 MHz were calculated by setting the *NOE*s to those at 600 MHz. As shown in the text as well as in Fig. S7 of the Supporting information, the $J(0)$ function is quite insensitive to inconsistencies in the *NOE*s, hence this choice is not problematic. *Top* correlation plots with a diagonal at $y = x$. *Bottom* distributions of the ratios

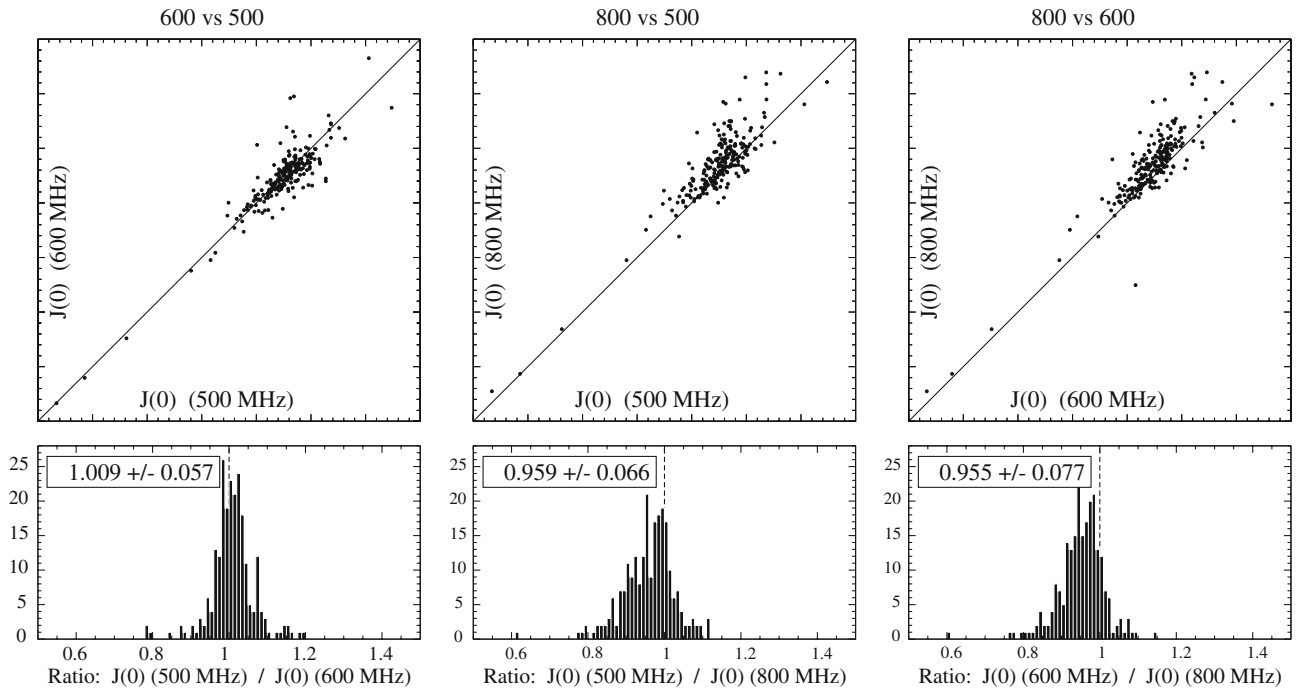

Fig. 2 $J(0)$ consistency of datasets for PSE-4. The consistency of both 500 and 600 MHz data is high, whereas the consistency of the 800 MHz data is lower. *Top* correlation plots with a diagonal at $y = x$. *Bottom* distributions of the ratios

Supporting information). This observation is valid at least for S^2 varying from 0.6 to 1 and τ_m varying from 5 to 40 ns, thus valid for most protein NMR studies. Additionally, variations in the local correlation time τ_e (from 20 to 100 ps) do not affect the field independent nature of the functions (data not shown), a prerequisite for their use as consistency tests. Contrary to the proposed tests, the normalisation of R_2 by the square root of the magnetic field $(R_2/\sqrt{B_0})$ does not create a field independent function unaffected by local dynamics (data not shown) and, thus, should not be used for consistency validation of multiple field R_2 data.

A second prerequisite for the use of these functions as consistency tests is that these be affected by inconsistencies. Tables 2, 3 and 4 indeed show that errors in the transverse relaxation rates affect the results of consistency testing and that both R_1 and *NOE* have a negligible effect on the calculated values for the three tests (see also Figs. S7–S12 in the Supporting information). Moreover, this effect of R_2 on the consistency functions is almost quantitative, even though we propose that assessment of consistency be performed in a qualitative manner, looking at the different vectors of a macromolecule as a whole, since particular conditions could give rise to a local deviation in R_2 unrelated to consistency issues. For example, errors on the measured rates will slightly affect consistency of single spins. However, taken as a whole, a consistent dataset with a non null error on the measured rates should give rise to a distribution of ratios centered at one, but with a spread proportional to the experimental error.

Of course, the proposed tests are not strictly perfect, i.e. some deviations are observed in the case of perfect datasets, for example when τ_m is short ($\sim$5 ns) probably because $\omega_H\tau_m$ approaches one where some assumptions are not valid anymore. However, these deviations are less than 1.5% in all cases (see Table 1). Moreover, some effects of R_1 and *NOE* inconsistencies can also be observed, mostly lower than 2.5% for 10% inconsistencies, thus not

Table 1 Simulations for consistent synthetic data for $S^2 = 0.8$ and $\tau_e = 20$ ps

τ_m (ns)	(500−600)/600 (%)			(500−800)/800 (%)			(600−800)/800 (%)		
	$J(0)$	F_η	F_{R_2}	$J(0)$	F_η	F_{R_2}	$J(0)$	F_η	F_{R_2}
5	−0.1	−0.1	0.7	−0.1	−0.1	1.4	−0.1	−0.1	0.7
10	0.0	0.0	0.2	0.0	0.0	0.5	0.0	0.0	0.2
20	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.1
40	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 2 Simulations for synthetic data (with inconsistent $R_1^{inc.} = R_1 + 10\%$) for $S^2 = 0.8$ and $\tau_e = 20$ ps

τ_m (ns)	(500−600)/600 (%)			(500−800)/800 (%)			(600−800)/800 (%)		
	$J(0)$	F_η	F_{R_2}	$J(0)$	F_η	F_{R_2}	$J(0)$	F_η	F_{R_2}
5	−2.3/1.7	−0.2/0.0	0.8/0.6	−2.4/1.0	−0.3/−0.1	1.6/1.4	−1.8/1.0	−0.2/0.0	0.8/0.7
10	−0.7/0.5	−0.1/0.0	0.3/0.2	−0.7/0.3	−0.1/0.0	0.5/0.4	−0.5/0.3	−0.1/0.0	0.3/0.2
20	−0.2/0.1	0.0/0.0	0.1/0.1	−0.2/0.1	0.0/0.0	0.2/0.1	−0.1/0.1	0.0/0.0	0.1/0.1
40	−0.1/0.0	0.0/0.0	0.0/0.0	−0.1/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0

Results are presented for inconsistencies in both low and high field data (low field/high field)

Table 3 Simulations for synthetic data (with inconsistent $R_2^{inc.} = R_2 + 10\%$) for $S^2 = 0.8$ and $\tau_e = 20$ ps

τ_m (ns)	(500−600)/600 (%)			(500−800)/800 (%)			(600−800)/800 (%)		
	$J(0)$	F_η	F_{R_2}	$J(0)$	F_η	F_{R_2}	$J(0)$	F_η	F_{R_2}
5	12.2/−10.5	10.1/−9.2	10.6/−8.4	12.2/−10.1	10.0/−9.3	11.4/−7.7	11.7/−10.0	10.0/−9.2	10.7/−8.4
10	10.7/−9.5	10.0/−9.1	10.2/−8.9	10.7/−9.4	10.0/−9.1	10.5/−8.7	10.5/−9.4	10.0/−9.1	10.2/−8.9
20	10.2/−9.2	10.0/−9.1	10.1/−9.0	10.2/−9.2	10.0/−9.1	10.1/−9.0	10.1/−9.2	10.0/−9.1	10.1/−9.0
40	10.1/−9.1	10.0/−9.1	10.0/−9.1	10.1/−9.1	10.0/−9.1	10.0/−9.1	10.0/−9.1	10.0/−9.1	10.0/−9.1

Results are presented for inconsistencies in both low and high field data (low field/high field)

Table 4 Simulations for synthetic data (with inconsistent $NOE^{inc.} = NOE + 0.1$) for $S^2 = 0.8$ and $\tau_e = 20$ ps

τ_m (ns)	(500−600)/600 (%)			(500−800)/800 (%)			(600−800)/800 (%)		
	$J(0)$	F_η	F_{R_2}	$J(0)$	F_η	F_{R_2}	$J(0)$	F_η	F_{R_2}
5	0.2/−0.3	0.4/−0.4	0.2/1.1	0.2/−0.2	0.3/−0.4	1.0/1.7	0.2/−0.2	0.3/−0.3	0.4/1.0
10	0.1/−0.1	0.2/−0.1	0.0/0.3	0.1/−0.1	0.1/−0.1	0.3/0.5	0.0/0.0	0.1/0.1	0.1/0.3
20	0.0/0.0	0.0/0.0	0.0/0.1	0.0/0.0	0.0/0.0	0.1/0.2	0.0/0.0	0.0/0.0	0.0/0.1
40	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0

Results are presented for inconsistencies in both low and high field data (low field/high field)

precluding the use of these tests for consistency testing. Of course, if different inconsistencies are combined, these effects also combine in an additive way, potentially increasing or decreasing the consistency scores (depending on the combination), but again in a limited way since the effects on $J(0)$, F_η and F_{R_2} of both R_1 and *NOE* inconsistencies are small (see Tables 2, 4 as well as Figs. S7–S9 in the Supporting information).

Hence, the above arguments confirm the availability of these functions for use as consistency tests. However, as all three functions report on the same inconsistency (affecting R_2), we prefer the use of the spectral density at zero frequency $J(0)$ alone since it does not rely on an estimation of the global correlation time, neither on a measure of θ, the angle between the ^{15}N–^{1}H vector and the principal axis of the ^{15}N chemical shift tensor. Hence, $J(0)$ is less likely to be affected by incorrect parametrisation of input parameters. For example, an error of 10% in the estimation of τ_m (which certainly cannot be excluded) gives rise to an apparent inconsistency of up to 2% for perfect data in both F_η and F_{R_2} (data not shown). For these reasons, we will focus solely on consistency assessment using $J(0)$ in the examples discussed below.

Consistency of experimental datasets

Since, at short correlation times (i.e. <5 ns), consistency tests are less efficient, only datasets for which the correlation time was estimated to be ≥5 ns were extracted (see Table 5 for a description of the method used to estimate τ_m). Among the datasets retrieved from the BMRB (Ulrich et al. 2008), some showed apparent inconsistencies, whereas others displayed excellent multiple field consistency (see Table 5).

Examples of very good consistency included data for the enzyme human neutrophil gelatinase-associated lipocalin (BMRB 4267, Coles et al. 1999), the apo-celular retinol-binding protein type I (BMRB 5331, Franzoni et al. 2002),

Table 5 Consistency results for data retrieved from the BMRB

τ_m^{app} (ns)[a]	Residues	Temperature (°C)	$J(0)$ Test			BMRB
			(500−600)/600 (%)	(500−800)/800 (%)	(600−800)/800 (%)	
5.4	100	25.0	4.0	–	–	5079
6.4	100	25.0	8.6	–	–	5080
6.6	102	25.0	−11.6	–	–	5687
7.1	135	25.0	−1.7	–	–	5330
7.5	135	25.0	3.5	–	–	5331
7.6	102	25.0	−13.4	–	–	15437
8.2	139	25.0	−0.9	–	–	5841
8.4	128	16.0	−0.6	−2.0	−1.5	6243
10.5	214	30.0	–	–	0.2	5746
10.9	139	22.0	−9.2	–	–	6474
11.3	179	25.0	0.1	1.7	2.1	4267[b]
11.4	271	31.5	0.9	−4.1	−4.8	6838
11.5	144	20.0	33.7	–	–	15445
11.6	263	30.0	0.3	2.2	2.0	16392
27.4	2 × 248	20.0	–	–	4.8	15066
29.4	2 × 248	20.0	–	–	−4.8	15065
30.1	2 × 248	20.0	–	–	−4.5	15064

[a] The apparent correlation time τ_m^{app} is estimated from the relation $\tau_m^{app} = 2.5\ J(0)$ using the mean value for $J(0)$ (Fischer et al. 1998)

[b] Data at the highest magnetic field was from 750 MHz, not 800 MHz

the enzyme adenylate kinase in complex with inhibitor Ap5A (BMRB 5746, Shapiro et al. 2002), the kinase-interacting FHA domain of KAPP protein (BMRB 5841, Ding et al. 2005), the protein azurin (BMRB 6243, Zhuravleva et al. 2004) and the class A β-lactamase TEM-1 (BMRB 16392, Savard and Gagné 2006).

On the contrary, some datasets displayed extreme apparent inconsistencies. These include data from BMRB accession number 5687 (Go et al. 2008), 6474 (Ding et al. 2005), 15437 (Go et al. 2008) and 15445 (Soss and Flynn 2007) where inconsistencies were $\geq$10% when comparing data acquired at 500 and 600 MHz. BMRB 5687 and 15437 both belong to the same study by Go et al. (2008) where the two proteins display similar apparent inconsistency with either R_2 at 600 MHz being over-estimated or, inversely, R_2 at 500 MHz being under-estimated. These inconsistent R_2 may have lead to R_{ex} of same magnitude as R_2 and unusually large S^2 errors for several residues in both S-836 (BMRB 15437) and S-824 (BMRB 5687). In the case of S-836 (BMRB 15437), most R_{ex} are co-localised and have significant values, indicating the potential consistency of this dataset which could probe a protein with important µs–ms motions. On the contrary, model-free analysis results for protein S-824 (BMRB 5687) display more scattered R_{ex} parameters of low value. However, since the authors only analysed the details of residues affected by R_{ex} of significant values ($>2s^{-1}$), the possible effect of inconsistencies for protein S-824 (BMRB 5687) are diminished for what concerns data interpretation. Hence, in this case, even though raw consistency tests show that inconsistencies are present, the authors took the necessary precautions to minimise artifacts.

BMRB 6474 is a very interesting case since these data relate to the bound state of the protein probed in BMRB 5841, which displayed very good consistency. Hence, as proposed in the study by Ding et al. (2005), this could be a very good example of conformational exchange caused by ligand binding. Hence, for BMRB 6474, the apparent inconsistency would be artificially caused by µs–ms motions (as confirmed by relaxation dispersion experiments Ding et al. 2005).

BMRB 15445 is different; in this case, the authors associated a R_{ex} term to 15 residues out of 144 (Soss and Flynn 2007). Removing these residues from the consistency analysis yields a similar inconsistency score of 33.9% (the highest inconsistency we could analyse in this study). Since the average R_2 is too high at 500 MHz compared to 600 MHz (R_2 were $\sim$20% higher at 500 MHz compared to 600 MHz), it is, however, normal that few R_{ex} terms were introduced in the analysis. In this case, this is the global tumbling description which may be incorrect since, for example, the τ_m^{app} (see Table 5 for a description of the method used to estimate τ_m) at 500 and 600 MHz were, respectively, 13.1 and 9.8 ns. It is probable that the

authors did not notice this problem when looking at the details of local dynamics. In fact, the authors chose an isotropic diffusion tensor despite a D_{ratio} ($D_{\|}/D_{\perp}$) of 1.1. It is not clear what effects this incorrect definition of the global diffusion introduced. The combination of this incorrect characterisation of global tumbling and over-estimated R_2 at the low field would be suggested to give rise to two-timescale motions, which is apparently not the case (Soss and Flynn 2007). It is clear that consistency tests, in this case, could have helped to improve the analysis.

Moderate inconsistencies, on the other hand, were observed for some datasets including BMRB accession numbers 5079 (Reincke et al. 2001), 5080 (Reincke et al. 2001), 5331 (Franzoni et al. 2002), 6838 (Morin and Gagné 2009), 15064 (Kempf et al. 2007), 15065 (Kempf et al. 2007) and 15066 (Kempf et al. 2007). Datasets 5079 and 5080 belong to the same study and are respectively for the reduced and oxidised forms of cytochrome c_{552} (Reincke et al. 2001). For both datasets, consistency tests indicate a significantly higher R_2 at 500 MHz compared to that at 600 MHz. The authors, in this case, may have missed important dynamic features of their protein which could have helped explain, for example, their hydrogen exchange data.

The dataset for holo-cellular retinol-binding protein type I (BMRB 5331, Franzoni et al. 2002) shows a low apparent inconsistency of 3.5% for data recorded at 500 and 600 MHz. The apo form of the same protein (BMRB 5330, also from the same study) showed high consistency. As in the case of BMRB 5079 and 5080 (Reincke et al. 2001), consistency tests indicate a higher R_2 at 500 MHz compared to that at 600 MHz which strongly prevents the appearance of R_{ex} terms in the model-free analysis. Of course, this inconsistency could cause other problems such as incorrect characterisation of the global tumbling or aberrant S^2 values. However, its small amplitude may not affect data analysis. In the holoprotein, multiple sets of resonances were observed for only eight residues, but these were attributed to chemical micro-heterogeneity in the NMR sample, and therefore would not affect relaxation data in a field-dependent manner.

The datasets for the triosephosphate isomerase dimer (BMRB 15064, 15065 and 15066) also show apparent inconsistencies. However, BMRB 15064 and 15065 show apparent inconsistencies in opposite directions compared to BMRB 15066, consistent with the findings of Kempf et al. (2007) who assessed that the wild-type protein (BMRB 15064 and 15065) was affected by μs–ms motions in loops six and seven, whereas the mutant (BMRB 15066) was not. Considering these data, these results perfectly make sense. However, for BMRB 15066, R_2 were lower than expected at 800 MHz. Hence, whereas their data for the wild-type protein (BMRB 15064 and 15065) appear of high quality with μs–ms motions co-localising on the 3D structure, it is possible that data for the mutant (BMRB 15066) are slightly inconsistent and mask the underlying dynamics.

It is difficult from consistency tests alone to decide whether or not the apparently inconsistent datasets are indeed erroneous. An apparent low consistency could be caused by widespread slow μs–ms motions affecting R_2 in a field dependent manner. However, such low consistency results indicate the need for a more in-depth lookup of obtained results in light of other information on the actual system studied (see below for such an analysis for proteins TEM-1 and PSE-4). Additionally, inconsistencies can be revealed by consistency tests, but be hidden because of limitations in analytical schemes often used for model-free analysis, particularly in the case of model selection (d'Auvergne and Gooley 2003).

It should be noted that the low number of retrieved datasets highlights a gap between the publication of relaxation studies and the deposition of the related experimental relaxation data within the BMRB (Ulrich et al. 2008). We would like to encourage NMR spectroscopists to deposit their relaxation data within the BMRB for access of these data by the scientific community. These consistency tests should be performed prior to data deposition in the BMRB with notes deposited as well to explain the obtained results, either for high or low consistency.

Class A β-lactamases TEM-1 and PSE-4 as test cases for inconsistent R_2

Two studies were performed on very similar systems in our laboratory. Datasets differed in their respective level of consistency, making these studies a particularly good test case for consistency tests. TEM-1 dataset (Savard and Gagné 2006) displayed a very high consistency leading to a straightforward analysis of relaxation data. However, PSE-4 datasets (Morin and Gagné 2009) were less consistent with each other, leading to an analysis scheme in the model-free formalism needing the removal of the aberrant data. Here, we will focus on the differences of these two studies and on the advantage consistency tests gave for PSE-4 data analysis.

TEM-1 (BMRB 16392)

β-Lactamase TEM-1 was studied by recording spin relaxation data at three magnetic fields: 500, 600 and 800 MHz (Savard and Gagné 2006). The consistency of these datasets was high (see Fig. 1; Table 5), which allowed the analysis within the model-free formalism using all data recorded. From this analysis, 12 N–H vectors required a R_{ex} in their selected model-free model. These were mostly

clustered on the 3D structure co-localising with two N–H vectors with extremely broadened resonances, supporting their real existence. Moreover, the low number of R_{ex} for this 263 residue protein was consistent with a very high quality HSQC spectrum with sharp peaks apparently not affected by broadening from conformational exchange.

Even though consistency tests should be used to assess consistency of data for single residues, they can also be useful in identifying residues for which dynamics are of particular interest or, on the contrary, for which data is erroneous, potentially from peak overlap or other reason. Such an outlier is present in the $J(0)$ correlation plots for TEM-1 (see Fig. 1). This residue is Thr141 which was not assigned to any model-free model in the original work of Savard and Gagné (2006) who used the analytical scheme of Mandel et al. (1995) based on F-test statistics. However, in a recent re-analysis of these datasets (Fisette et al. Biophys J, in press) using *relax* (d'Auvergne and Gooley 2008a, b) and an analytical scheme involving small sample size Akaike information criterion (AIC_c, Hurvich and Tsai 1989), this residue was assigned to model $m2$. However, in this case, the fit was of poor quality, with a χ^2 value of 24.9, i.e. the second highest score for TEM-1. Hence, the $J(0)$ test would have been useful in this case for identifying this residue and either fix its data or exclude it from the analysis to avoid the extraction of aberrant information.

PSE-4 (BMRB 6838)

An NMR study on the dynamics of the class A β-lactamase PSE-4 was recently presented (Morin and Gagné 2009) in which we discussed the inconsistency of some data among a multiple magnetic field dataset (see Fig. 2; Table 5). In summary, from a three magnetic field dataset (500, 600 and 800 MHz), the R_2 at 800 MHz were identified as inconsistent and discarded prior to model-free analysis.

In fact, including R_2 at 800 MHz caused dozens of artifactual R_{ex} appearing all over the protein. Removing the inconsistent data solved the problem and high quality dynamic information could then be extracted. However, removing other parts of the dataset would not solve the problem of the presence of widespread slow µs–ms motions (see Table 6). Hence, the $J(0)$ test for R_2 consistency could easily detect the inconsistent R_2 at 800 MHz, whereas other reporters such as the χ^2 value were useless for identifying the inconsistent data. In the end, the model-free analysis published for PSE-4 included 20 R_{ex} parameters, among which five had especially high values and co-localised in the active site where broadened resonances were also observed. This is similar to what was obtained for the homologous protein TEM-1, and very different from what would have been obtained if the aberrant data had been included in the analysis.

This example clearly shows the advantage of using consistency tests to avoid the time consuming step of fitting model-free parameters to different combinations of subsets of the complete dataset in order to determine if aberrant data are present (as were performed here, for test case purposes, see Table 6).

Minimizing inconsistencies

Using the tests presented above, inconsistencies can be detected so they are not incorporated into a joint multiple magnetic field data analysis. However, it is best to minimise the potential for inconsistencies before actual recording of datasets, so recorded data can be used in totality in the

Table 6 Model-free optimisation for PSE-4 using different datasets

Datasets	$< \text{local } \tau_m >$ (ns)	$< S^2 >$	$< \chi^2 >$ [a]	Number of R_{ex}
All	12.55 ± 1.16	0.86 ± 0.09	0.77 ± 0.77	46
All but 500 MHz	12.57 ± 1.69	0.84 ± 0.10	0.50 ± 0.62	42
All but 600 MHz	12.49 ± 1.28	0.86 ± 0.08	0.61 ± 0.49	49
All but 800 MHz	12.57 ± 1.24	0.86 ± 0.09	0.57 ± 0.49	9
All but R_1 800 MHz	12.53 ± 1.19	0.86 ± 0.09	0.72 ± 0.77	45
All but R_2 800 MHz	12.64 ± 1.11	0.86 ± 0.09	0.76 ± 0.77	8
All but *NOE* 800 MHz	12.52 ± 1.23	0.86 ± 0.08	0.68 ± 0.54	46
Only 500 MHz[b]	12.52 ± 1.17	0.88 ± 0.07	0.15 ± 0.21	0
Only 600 MHz[b]	12.75 ± 1.28	0.87 ± 0.07	0.13 ± 0.20	0
Only 800 MHz[b]	13.11 ± 1.45	0.87 ± 0.06	0.30 ± 0.52	2

230 residues with data at the three magnetic fields were minimised using a local τ_m (model-free models *tm*0 to *tm*9 in *relax*, d'Auvergne and Gooley 2008a, b)

[a] The χ^2 values were normalised by dividing the obtained value by the number of datasets used

[b] Only model-free models *tm*0, *tm*1, *tm*2, *tm*3 and *tm*9 (i.e. with three parameters or less) were used for these small datasets

subsequent analysis. There are three main factors one should pay attention to when recording R_1, R_2, and *NOE*: the sample itself, the effective temperature and pulse sequence parameters. Of course, these are inter-related.

Sample

Obviously, the sample should be in the same conditions throughout the complete experimental scheme. Concentration, in the case of using multiple samples, should be carefully matched as it affects the solvent viscosity, which in turn affects the global tumbling of the molecule (for example, in the absence of dimerisation, we observed a 5% modulation of the apparent correlation time for PSE-4 when the protein concentration was changed from 0.125 to 0.5 mM, unpublished data). Sample integrity, of course, should also be monitored as a macromolecule being degraded as a function of time will, among other problems, have its global correlation time being modulated, hence affecting datasets consistency.

In the case of the use of a single sample for the whole experimental scheme, a good way to assess sample integrity along time is to record a complete spin relaxation dataset at one magnetic field and, after data at other magnetic fields are recorded, record the first dataset again. This allows the comparison of the relaxation data at the start and end of the experimental scheme, allowing the detection of any change which could affect dynamics that may not be detected by $^{1}H–^{15}N$ 2D correlation spectra. Moreover, this allows a direct assessment of experimental error. This approach was adopted in our study of PSE-4 (Morin and Gagné 2009).

In the case of multiple samples, special care must be taken to match both sample and salt concentrations as well as pH. As a check for these, relaxation data could be acquired at a common magnetic field for the different samples, allowing the comparison of datasets to assess the consistency of the samples used, prior to recording of data using other magnetic fields.

Temperature

As temperature affects the solvent viscosity, which in turn affects the diffusion of macromolecules in solution, temperature should be calibrated using standard procedures. Indeed, the Einstein relation (or Einstein–Smoluchowski relation) clearly shows the dependence of the rotational diffusion D_r on both the temperature T and the viscosity η_{visc} with $D_r = k_B T/f_r = 1/6\ \tau_m$ where k_B is the Boltzmann constant and $f_r = 8\pi\eta_{visc}R^3$ is the rotational frictional drag coefficient for a sphere of radius R (Einstein 1905; Smoluchowski 1906). Typically, the chemical shift separation of OH and CH resonances in methanol (CH_3OH) or ethylene glycol ($HOCH_2CH_2OH$) can be used for calibrating temperature. This is extremely important as temperature controllers within different probes generally behave differently depending on the probe vendor, model and/or generation, leading to differences in the effective temperature. See Fig. 3 for an example of such differences between temperature controllers of different probes.

Pulse sequence parameters

Pulse sequence details should be carefully matched with special care for solvent suppression as differences can severely influence exchanging amides (e.g. solvent exposed amides).

Heat compensation schemes should be used when recording experiments potentially causing sample heating, such as R_2 measurements, to avoid any internal inconsistency again arising from temperature variations. These inconsistencies are also caused by duty cycle variations which affect probe tuning and lead to non-ideal RF pulses, hence causing a decrease in internal consistency and, indirectly, in overall consistency. These problems are increased at high magnetic field and/or short recycle delays. See Yip and Zuiderweg (2005) for a discussion on this topic.

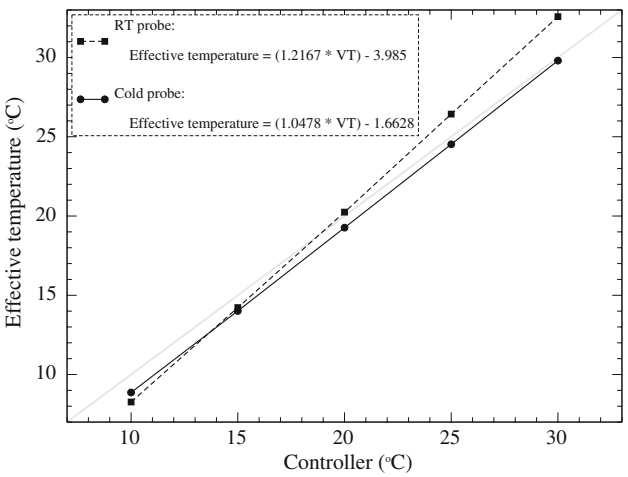

Fig. 3 Temperature calibration of different probes. Effective temperature (calibrated using methanol) as a function of the temperature set for the variable temperature (VT) controller within Varian RT (*broken line* and *squares*) and cold (*solid line* and *circles*) probes. The *gray line* indicates a perfect agreement between the controller temperature and the effective temperature. For the temperature range shown, a 4% CH_3OH/96%CD_3OD sample was used with the following relation: $T = (4.109 - \Delta)/0.008708$, where Δ is the chemical shift difference between the OH and CH_3 resonances (from Bruker Instruments VT-Calibration Manual). As can be seen, temperature on both probes is very linear ($r^2 > 0.9999$), but the slope is different, leading to potential differences in temperatures if calibration is not undertaken to assess the effective temperature in the sample

In the case of CPMG-based R_2 experiments, similar RF field strength during the CPMG pulse train should be used. The field strength should be high enough so that no important off-resonance effects arise within the datasets. However, the field strength should not be too high to avoid too much sample heating. A simple way to avoid such off-resonance effects could be the use of the modified phase cycling proposed by Yip and Zuiderweg (2004). Moreover, sample heating should be similar at all magnetic fields to avoid a modulation of R_2 (which would decrease as a function of temperature). Interleaving of the different delays in a scan by scan mode might help in stabilising the temperature within a single dataset and, indirectly, stabilise temperature throughout acquisition at multiple magnetic fields (Korzhnev et al. 2001). Finally, unnecessary long relaxation delays during such experiments should be avoided also to reduce sample heating.

Discussion

As is demonstrated using synthetic datasets, the proposed consistency tests are field independent functions suitable for assessing consistency of multiple magnetic field datasets. Since local variations in R_2 caused by slow µs–ms motions could arise, these tests should be used in a qualitative manner for assessing overall quality of datasets. Moreover, these tests could also be used to investigate the quality of data for individual bond vectors.

Obviously, N–H groups affected by µs–ms motions will appear inconsistent using tests for R_2 since R_{ex} is scaled quadratically with the strength of the magnetic field. However, proteins rarely display µs–ms motions throughout their whole sequence and a majority of residues should be unaffected by such motions. Hence, once again, we point to the need for a global qualitative analysis instead of a residue focused quantitative analysis. A better option for assessing datasets consistency of such systems would be the recording of R_{ex}-free R_2 (Hansen et al. 2007). Indeed, such experiments yield the exchange-free R_2 (by quenching the effect of exchange) which should then give rise to high consistency scores in favorable conditions, irrespective of whether there are or not µs–ms motions in the particular system under study.

High field effects could arise within relaxation data based on a modulation of the *CSA* constant c as a function of the magnetic field. Indeed, the *CSA* has been shown previously to vary depending on the actual N–H bond vector studied (Fushman et al. 1998; Kroenke et al. 1999; Tjandra et al. 1996a). Assuming a value of −172 ppm for all N–H bonds, as in most spin relaxation studies, could potentially give the impression of inconsistencies at high field for some residues where the *CSA* is significantly different from −172 ppm. However, this would most probably have the effect of broadening the distribution of ratios, but not of skewing it, thus conserving a fairly good consistency appearance overall. Hence, we believe this should not affect consistency tests too much.

Of course, it would also be very useful to have tests for the assessment of R_1 and *NOE* data consistency. However, it turns out very difficult, if not impossible, to develop field independent functions for consistency testing purposes of R_1 and *NOE* that would also be independent of local dynamics. Indeed, contrary to R_2, R_1 and *NOE* are interdependent and influenced by fast motions with negligible contribution from the very slow motions probed by $J(0)$, as shown in Eqs. 4, 5 and 6. In fact, performing consistency testing on R_1 and *NOE* would require the knowledge of local dynamics which is actually why the data is recorded.

We do not claim to be the first to propose ways to improve analysis of spin relaxation data. However, we think the use of these simple validation tests, in particular of $J(0)$, for the assessment of R_2 consistency within multiple field datasets will be very useful. In particular, it will reduce the extraction of erroneous µs–ms motions. Additionally, it will allow a better description of global tumbling for NMR spectroscopists using the Kay et al. (1989) R_2/R_1 approach to determination of the isotropic correlation time τ_m, hence contributing to a better description of local motions. Overall, the use of these tests will be a valuable addition to the set of analytical tools available for spin relaxation data.

Conclusions

From the limited number of published experimental datasets used here, it is clear that some bias can sometime appear in spin relaxation studies even though great care is taken to avoid such problems. This was the case in a recent work on protein PSE-4 by our laboratory (Morin and Gagné 2009) where consistency tests helped in removing inconsistent data to allow a reliable description of protein dynamics.

We thus believe the introduction of consistency tests within the analytical scheme of spin relaxation data could allow a better description of the systems studied. Indeed, when inconsistencies are seen, datasets could be excluded if every other data indicates no widespread µs–ms motions are present. On the contrary, the tests could also indicate a particular situation where a macromolecule has widespread conformational exchange. Hence, high consistency would confirm the data is of good quality and the macromolecule is not affected by widespread R_{ex}, while low consistency would require a deeper look at the data. Hence, consistency testing should be performed prior to any joint analysis of

different datasets. This includes multiple field datasets, as well as datasets recorded with different samples, such as in the case of unstable samples.

The tests presented here apply to ^{15}N spin relaxation data, mainly R_1, R_2 and *NOE*, for the identification of inconsistencies within R_2. However, the same principles could apply to other spins and ought to be used by anyone requesting high quality data being extracted from multiple field datasets. These tests are now available within the program *relax* (versions 1.2.14 and higher) (d'Auvergne and Gooley 2008a, b).

Supporting information

Plots of synthetic datasets and corresponding consistency tests are available as supporting information.

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