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Potential bias in NMR relaxation data introduced by peak intensity analysis and curve fitting methods

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Abstract

We present an evaluation of the accuracy and precision of relaxation rates calculated using a variety of methods, applied to data sets obtained for several very different protein systems. We show that common methods of data evaluation, such as the determination of peak heights and peak volumes, may be subject to bias, giving incorrect values for quantities such as R_1 and R_2. For example, one common method of peak-height determination, using a search routine to obtain the peak-height maximum in successive spectra, may be a source of significant systematic error in the relaxation rate. The alternative use of peak volumes or of a fixed coordinate position for the peak height in successive spectra gives more accurate results, particularly in cases where the signal/noise is low, but these methods have inherent problems of their own. For example, volumes are difficult to quantitate for overlapped peaks. We show that with any method of sampling the peak intensity, the choice of a 2- or 3-parameter equation to fit the exponential relaxation decay curves can dramatically affect both the accuracy and precision of the calculated relaxation rates. In general, a 2-parameter fit of relaxation decay curves is preferable. However, for very low intensity peaks a 3 parameter fit may be more appropriate.

Abbreviations: apoMb, apomyoglobin; DHFR, dihydrofolate reductase; G121V DHFR, mutant of DHFR where glycine at position 121 has been substituted by valine; Fv, variable domain fragment of the catalytic antibody NPN43C9; PrP(29-231), prion protein, residues 29-231; NOE, nuclear Overhauser effect.

Introduction

NMR is a powerful tool for probing molecular motion on various time-scales in proteins. Knowledge of backbone and side-chain dynamics complements structural studies and can give insight into thermodynamic stability, folding, molecular recognition and catalysis (Palmer, 1993; Dayie et al., 1996; Palmer, 1997). The 'Model Free' formalism (Lipari and Szabo, 1982a,b) is commonly used to identify a combination of motional parameters that best describes the measured relaxation data. Statistical tests are used extensively to discriminate between different combinations of parameters and these tests rely upon the uncertainties in the measured relaxation data. Clearly, the correct interpretation of NMR relaxation data in terms of molecular motion is heavily dependent upon obtaining accurate estimates of relaxation rates and realistic estimates of the associated uncertainties.

The NMR relaxation data used to probe molecular motions in proteins are typically the $\{^1H\}$-^{15}N NOE and ^{15}N R_1 and R_2 relaxation rates. The NMR experiments used to measure these rates are well described in the literature (reviewed in Palmer, 1997). They consist of a series of ^1H-^{15}N HSQC experiments in which the

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number of counts of a loop in the pulse program is varied, leading to different evolution times ('delays') in the spectra. The spectra are analyzed as a function of the modulation created by the different relaxation delays.

Relaxation rate data are extracted from the NMR spectra by evaluation of the signal intensity for each peak for every time delay. Types of fits and peak picking to be used in the evaluation of 1D relaxation data have been extensively studied (Weiss and Ferretti, 1985a,b), and the optimal position of sample points along the decay curve has recently been discussed (Jones, 1997). Several methods are available for extraction of peak intensities from NMR spectra. These include: (1) peak volume integration; (2) peak height measurement at a fixed position; (3) the summing of a small grid of peak heights centered around the peak maximum; and (4) the measurement of peak height using a search for signal maximum. These methods are illustrated in Figure 1.

The ^{15}N R_1 and R_2 relaxation rates are determined by fitting a mono-exponential equation to volumes or heights of a given peak in a series of spectra recorded with different delays. The equation describes the intensity of the signal as a function of the delay time and contains two or three parameters. A 2 parameter fit (Equation 1) determines values for the decay rate, R, and for the intensity at time zero, I_0. A 3 parameter fit (Equation 2) adds an extra parameter for the offset, I_∞.

$$I(t) = I_0 e^{(-Rt)} \qquad \text{2 parameter fit,} \tag{1}$$

$$I(t) = I_0 e^{(-Rt)} + I_\infty \quad \text{3 parameter fit.} \tag{2}$$

A 2 parameter fit is appropriate when the NMR spectrum does not contain artifacts such as systematic t_1 artifacts and baseplane roll. If the spectrum is not ideal, then it may be more appropriate to use a 3 parameter fit. One might conclude that, as experimental data will always contain some degree of spectral artifacts, it would always be most appropriate to fit the decay curves to a 3 parameter equation. We find, however, that this is far from being true, and that a careful choice of the fitting equation is necessary if accurate values are to be obtained for the relaxation rates. Serious systematic errors and bias of results can occur in the acquisition, evaluation and processing of NMR relaxation spectra, if published methods are used without care. This paper describes the sources of some of these problems, and makes suggestions as to how they can be circumvented.

One potential source of inaccuracy in NMR relaxation time measurements is the variation in peak position caused by small changes in temperature. This problem is particularly acute for amide proton resonances, which are strongly temperature dependent. In order to avoid any difference in the position of the cross peaks in successive spectra, it is crucial to maintain the sample temperature as constant as possible. This can be done by applying temperature compensation methods (Wang and Bax, 1993) and by interleaving experiments with different delays (Tjandra et al., 1996). In general, however, problems with data acquisition are overshadowed by potential sources of bias in the extraction of intensity data from the spectra and in the fitting of the data to exponential rate equations.

The process of evaluation of the signal intensity for each peak for every time delay may be the source of considerable bias and inaccuracy. The inaccuracies associated with commonly-used methods are illustrated in Figure 1. The first method of quantitation consists of an integration over the whole peak (Figure 1a). This is the method of choice for well-resolved peaks, but it is not reliable whenever there is any peak overlap. In practice, for most protein systems of interest, a significant amount of peak overlap occurs in 2D spectra, so that if data are to be extracted from overlapped peaks, some form of peak height estimation is made (Skelton et al., 1993). The peak height can be determined at a fixed position, as illustrated in Figure 1b. This method will only be reliable if there is no movement in the position of peaks due to, for example, temperature instability. An alternative method of peak height estimation is to sum the heights in a grid around the peak maximum, as illustrated in Figure 1c. This approximates the averaging of the noise obtained by measuring volumes and at the same time minimizes the effects of overlap. A common method of peak extraction (Stone et al., 1992), which is used in a number of available NMR software packages, measures the intensity as the maximum height within a fixed box around the peak (Figure 1d). In this paper we will show that bias can be introduced when using this method.

Results and discussion

Bias from evaluating peak intensity

We were first alerted to the possibility of the introduction of bias in the determination of relaxation rates

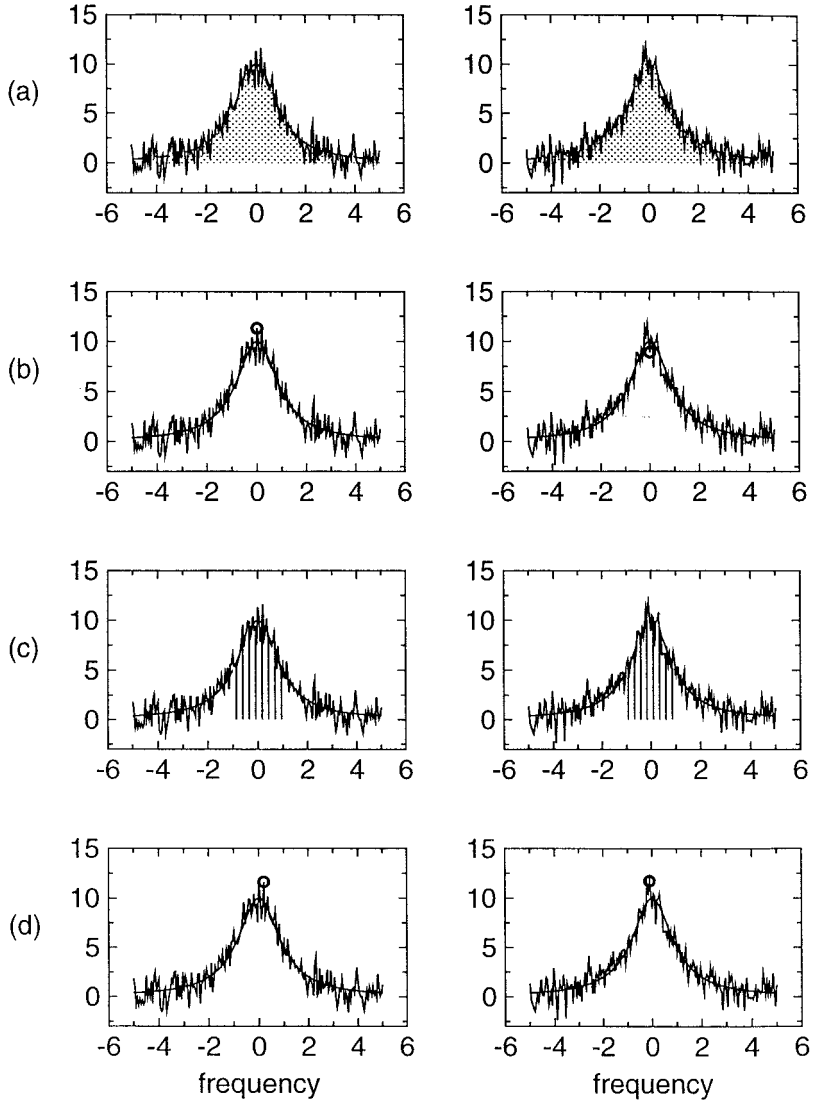

Figure 1. Simulated one-dimensional NMR data showing the effects of various methods of data extraction on the values extracted from relaxation data. A Lorentzian line shape (solid line) was calculated, and noise was added to it using a random number generator. All of the graphs (a)-(d) were derived from the same simulated signal, but the graphs on the left and on the right in each pair were derived using a different seed in the random number generator. (a) The intensity of the peak is extracted using the area under the peak (equivalent to the peak volume in multi-dimensional spectra). (b) The intensity of the peak is extracted as the peak height at a single fixed point. The values are seen to be quite different in the two cases: here the precision of the measurement will depend strongly on the noise, and does not give a good measure of the uncertainty in the signal height. (c) The intensity of the peak is estimated as the sum of values derived from a grid over the center of the peak. (d) The peak intensity is estimated as the observed maximum in a box around the peak. The measured value will be consistently higher than the signal, but the measured uncertainty will be a good measure of the difference in signal intensity between independent experiments.

when we observed a significant difference between rates obtained using a 2 or 3 parameter fit to experimental decay curves. To assess the extent to which the choice of a 2 or 3 parameter fit can affect the calculated rate, the values of the spin-lattice relaxation rates R_1 obtained from 2 and 3 parameter fits to peak heights were compared in six different proteins (Figure 2). Clearly, many residues show statistically significant differences; these arise when the 3 parameter fit introduces a significant offset. Using a positive offset in a 3 parameter fit results in a larger rate than the rate obtained from a 2 parameter fit to the same data, while a negative offset gives a smaller rate. In the systems studied most of the offsets are positive, suggesting that

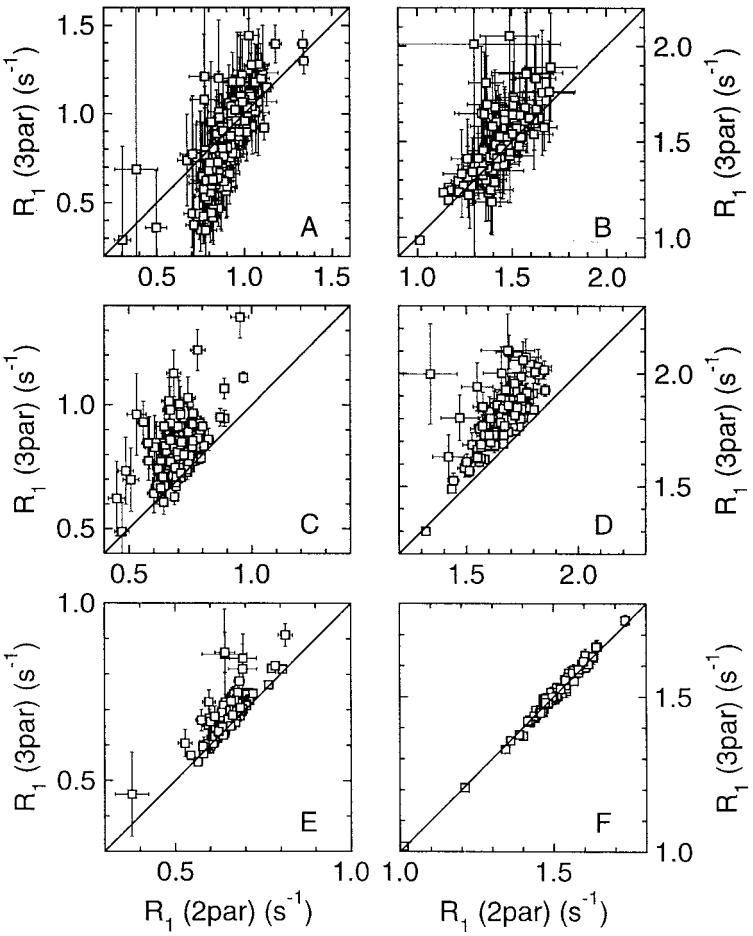

Figure 2. Comparison of R_1 values obtained from 2 and 3 parameter fits to peak heights. R_1 relaxation rates were measured using the pulse sequence of Farrow et al. (1994) implemented on Bruker AMX and DRX spectrometers at fields of 11.8, 14.1 and 19.0 T. Decay curves were fitted using the program CurveFit (Palmer et al., 1991). (A) 0.125 mM metallo-β-lactamase in 10 mM HEPES containing 0.01 mM $ZnCl_2$ and 0.01% NaN_3 at pH 7.0 and 22 °C (J.J.A.Huntley, H.J.Dyson, P.E.Wright, unpublished data). Decay sampled through 2 time constants. (B) 0.5 mM PrP(29-231) in 0.1 M sodium acetate buffer at pH 5.5 and 30 °C (Donne et al., 1997). Decay sampled through 3 time constants. (C) 1.0 mM DHFR in 70 mM potassium phosphate, containing 40 mM NADPH, 25 mM potassium chloride and 1 mM [^{2}H] DTT at pH 7.6, and 9 °C (Zaborowski et al., 2000). Decay sampled through 3.5 time constants. (D) 1.86 mM G121V DHFR in 50 mM potassium phosphate containing 11.5 mM folic acid, 100 mM potassium chloride, 1 mM EDTA and 1 mM [^{2}H] DTT at pH 6.8 and 33 °C (B.M. Duggan, H.J. Dyson, P.E. Wright, unpublished data). Decay sampled through 5 time constants. (E) 0.5 mM Fv with 0.08 mM *p*-nitrophenol in 10 mM tris containing 100 mM NaCl at pH 6.8 and 25 °C (Kroon et al., 1999). Decay sampled through 5 time constants. (F) 0.58 mM apoMb in 8 M urea at pH 2.3 and 20 °C (S. Schwarzinger, H.J. Dyson, P.E. Wright, unpublished data). Decay sampled through 8 time constants.

the 3 parameter fit can systematically overestimate the rates.

The systematic bias of the rates derived from 3 parameter fits is not the same for all proteins studied (Figure 2). It appears to be related to the extent to which the relaxation decay is sampled. Somewhat surprisingly, the least systematic bias is observed for those proteins whose decay was sampled for the smallest number of time constants (Figures 2A and 2B). As a greater number of time constants are sampled the systematic bias increases. An exception to this trend is the data set for urea-unfolded apoMb (Figure 2F), which show no apparent bias. This is due to the high signal to noise of the apoMb spectra, which allowed very accurate measurement of the intensities throughout the decay.

This bias apparently arises from the method by which the peak intensities are determined. The peak heights are measured by searching for a maximum within a specified box (Figure 1d). The drawback to this method of data estimation is that, for low intensity peaks, and for all peaks near the end of the decay, a

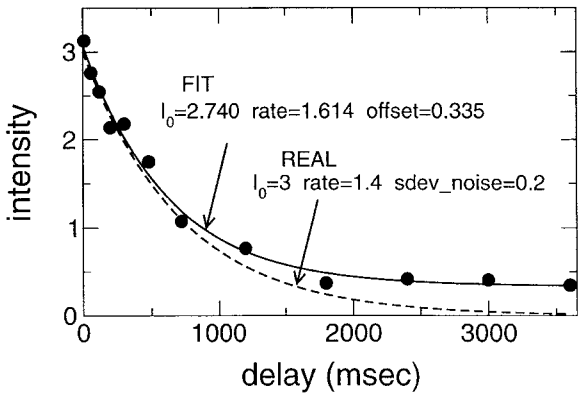

Figure 3. Simulated data set illustrating the problem that arises when method (d) of Figure 1 is used for low-intensity peaks. The dotted curve represents a calculated R_2 relaxation rate curve for a relaxation rate of 1.4 s^{-1}. Lorentzian lines were simulated for the delay times represented by the dots, and noise was added with a standard deviation of 0.2 units of the intensity scale. The intensity of the peak at each delay time was estimated using the observed maximum of the signal within a box (corresponding to Figure 1d), and these 'measured' values plotted and fitted with a 3-parameter fit (solid line). It can be seen that the rate derived from this calculation deviates significantly from the 'real' rate, by a factor of some 15%. The deviation can be minimized by leaving out the points at the longer delays: this is indicated by the coincidence of the solid and dotted lines at the lower decay times.

positive bias is introduced into the measurement, since the maximum within the box is always the highest-value point, which may represent the actual value of the data, but is much more likely to be representative of a noise peak. Thus, when the signal approaches zero, the measured peak intensity will never reach zero, as illustrated in Figure 3. Without appropriate corrective measures, this introduces a further set of serious bias problems in the estimations of relaxation rates from these data. One solution to this would apparently be to use a 3 parameter fit to compensate for this positive offset, but there is a problem with this approach. While only the heights towards the end of the decay (whose maxima are comparable to the noise maxima) are biased by the search routine, a 3 parameter fit applies a fixed offset throughout the entire decay curve, not just at the end. Therefore, using a 3 parameter fit to heights measured using a grid search for the maximum will produce incorrect rates because the points at the beginning of the decay are incorrectly compensated. Attempting to correct for the offset at the end of the decay by subtracting the mean maximum noise does not solve the problem. The heights towards the end of the decay will be closer to their true values, but the heights at the start of the decay, which are randomly reduced or increased due to the noise on which they are superimposed, will be uniformly reduced by the noise subtraction, resulting in inaccuracy in the evaluation of the decay rate.

Measuring volumes instead of a search for the peak height maximum should remove the problem of bias described above. Figure 4 compares the G121V DHFR R_1 rates obtained using peak volumes with rates obtained using peak heights. The R_1 values derived from a 2 parameter fit to heights are lower than the values derived from a 2 parameter fit to volumes because the heights are over-estimated at the end of the decay (Figure 4A). The mean R_1 obtained from a 2 parameter fit to volumes is 1.70 s^{-1}, while the mean R_1 from a 2 parameter fit to heights is 1.59 s^{-1}. The rates derived from a 3 parameter fit to heights show the opposite effect (Figure 4B). The mean R_1 from a 3 parameter fit to heights is 1.80 s^{-1}. The 6% difference in the rates is a significant source of systematic error because the average uncertainty of the rates is 2% for the 2 parameter fit and 5% for the 3 parameter fit. However, there is a very good correlation between the rates obtained from 2 and 3 parameter fits to volumes (Figure 4C) [much better than the correlation between rates derived from 2 and 3 parameter fits to heights (see Figure 2D)]. The mean R_1 obtained from a 3 parameter fit to volumes was 1.71 s^{-1}, very close to that obtained from a 2 parameter fit to volumes. Clearly, the measurement of peak volumes, rather than heights, is preferable wherever possible. However, most protein NMR spectra do not show sufficient dispersion for this method to be used reliably for all of the data. Therefore, peak heights must be used in order to utilize as much as possible of the data. We have determined a methodology for utilization of peak heights as data in the relaxation analysis in a manner that avoids the problems with data bias at the end of the decay.

A comparison was made of the rates derived from a 2 parameter fit to peak heights that are significantly larger ($> 2\times$) than the noise with the rates derived from a 2 parameter fit to volumes. This amounts to ignoring the peak heights that are comparable to the noise (i.e., those at later delays). The correlation plot (Figure 4D) shows that this treatment removes much of the bias introduced by the peak height search routine. The mean R_1 obtained in this case was 1.65 s^{-1}. Thus, the bias introduced by the necessity for using peak heights in spectra where a significant proportion of the cross peaks are overlapped, disallowing use of peak volume as a measure, can be eliminated simply

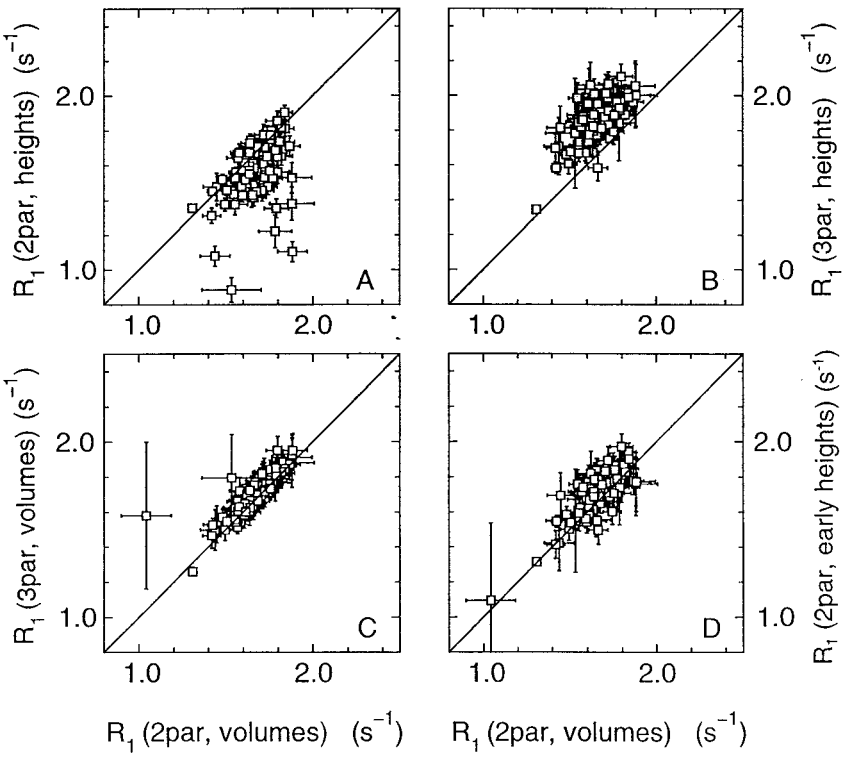

Figure 4. Comparison of G121V DHFR R_1 rates determined in different ways. (A) 2 parameter fit to volumes vs 2 parameter fit to heights. (B) 2 parameter fit to volumes vs 3 parameter fit to heights. (C) 2 parameter fit to volumes vs 3 parameter fit to volumes. (D) 2 parameter fit to volumes vs 2 parameter fit ignoring peak heights that are less than 2 times the height of the noise peaks (delays greater than three time constants in this case).

by ignoring data at the end of the decay where the signal becomes comparable to the noise level.

As a practical point, we have made a number of modifications to the standard computational methods available for quantitating relaxation data. A program (MakeCFfiles) that extracts the values of the peaks for each delay (as generated by NMRView (Johnson & Blevins, 1994)) was written so that one can easily define a point after which the values should not be considered. One can define, for example, a minimal level of twice the noise for peaks to be taken into consideration. The individual decay points for the different residues (which are submitted to the curve fitting procedure) will therefore have only points that stand above the designated signal to noise level. A program has been written (Curveview which is available to users as a perl/TK script) that allows visual inspection of the decay curves and the fitted rates, and has been found to be of great utility, allowing us to assess directly the quality of the curve fitting.

An alternative method to avoid bias at the end of the decay is to use a fixed position for the peak height. This approach will work assuming that the position of the peak does not change between spectra, due to for example, temperature instability. It is well known that the resonance of a non-hydrogen-bonded amide proton shifts approximately 8 ppb per K. Even after zero filling the cross peaks in a ^{15}N HSQC are customarily described by few data points and a peak shift of just one data point in this dimension would have a dramatic effect on peak height. Temperature instability can be a problem in the R_2 experiments due to the heating effects of the spin-lock pulse train. Precise temperature control is therefore crucial if a fixed peak position is to be used for data analysis.

Another alternative to the use of peak heights and peak volumes is to utilize a grid around each peak, where the peak heights within the grid box are summed to give the intensity. This can reduce the problem of peak overlap found when using volumes. It may also have the advantage of reducing the effect of small shifts in peak position. As the number of data points in the grid increases, variations in the noise at each data point in the grid are averaged. The mea-

sure of a small box around a peak is a compromise between a single peak height and a total volume with their respective advantages and problems. An increasing box size represents a continuum between the two approaches. There is a cut-off after which the increase in size of the grid actually has a detrimental effect on signal/noise. This is when peak intensity close to the base-plane is incorporated into the integration; here the effect is to add noise rather than signal into the measurement.

Accuracy and precision of relaxation decay curve fitting

Several factors have been identified that appear to influence the fitting of decay curves: the number of time constants through which the decay curve is sampled, the signal to noise ratio, and the offset. To test the influence of these factors when data are fitted to 2 or 3 parameters, we have simulated decay curves with two different values for each of these three factors. The simulated data were fitted with both the 2 and 3 parameter equations. All simulated decay curves had a rate of 1 s^{-1}. Table 1 shows the mean and the standard deviation of the initial intensities, rates, and offsets derived from fitting simulated decay curves of eight different combinations of an initial intensity (I_0), an offset (I_∞) and the number of characteristic time constants sampled (T). The standard deviations reported are not the uncertainties in the fitted parameters but a measure of the scatter of those fitted parameters. Thus, the means are a measure of the accuracy of the rates and the standard deviations are a measure of their precision.

Where there is no offset both 2 and 3 parameter fits have similar accuracy, but the precision of the 2 parameter fit is always better. Where there is an offset, the 2 parameter fits are inaccurate because the decay curve is forced to zero. Partial sampling of the decay ($T = 1$) does not affect the accuracy of the fitted rates, but dramatically decreases the precision of the values obtained from 3 parameter fitting. The precision of rates derived from weak peaks ($I_0 = 1$) is less than the precision of those derived from strong peaks ($I_0 = 10$). For weak peaks the deterioration in the precision is more profound for 3 parameter fits. The inaccuracy or bias in the 2 parameter fit is most pronounced for cases where the ratio I_∞/I_0 is largest. The simulations indicate that a 2 parameter fit is always more precise than the 3 parameter fit. If there is an offset, however, the 2 parameter fit is less accurate.

To obtain experimental relaxation rates with the highest accuracy and precision possible the point must be determined at which the improved precision of a 2 parameter fit is outweighed by the inaccuracy caused by the presence of an uncorrected offset. To address this point, we simulated another series of relaxation decay curves with rates of 1 s^{-1}, an initial intensity of 1 and an increasing positive offset. The simulated data points were fitted with 2 and 3 parameter equations. The rates determined using a 2 or 3 parameter fit can be plotted *versus* the ratio of the offset to the initial intensity (I_∞/I_0). As would be expected, the rate determined using a 3 parameter fit remains constant and is unaffected by the presence of an offset, while the value obtained from the 2-parameter fit decreases as I_∞/I_0 increases. Thus, although the precision of the rate obtained from a 2 parameter fit is always better than that obtained from a 3 parameter fit (Table 1), the rate becomes less accurate as the offset increases. There is a point at which the inaccuracy of the rate derived from the 2 parameter fit is greater than the decrease in precision of the rate derived from a 3-parameter fit. This cutoff point is when the difference in the rates determined from 2 or 3 parameter fits becomes greater than the sum of their uncertainties. At this point, a 3 parameter fit should be used. The preference for a 3-parameter fit over a 2-parameter fit must be evaluated on a residue-by-residue basis. In general, I_∞/I_0 is small for the more intense peaks; in these situations a 2 parameter fit is most appropriate. For weak peaks, where the initial intensity is small relative to the base-plane noise and I_∞/I_0 can become large, a 3 parameter fit is more likely to be appropriate. Most typically, a 3 parameter fit might be preferred over a 2 parameter fit for transverse relaxation of well resolved, rapidly decaying resonances, for example, those exhibiting broadening due to exchange.

F-tests have frequently been used to decide if a 2 or 3 parameter fit best describes the data (Bhattacharya et al., 1999). However we have found that the F-test should be used with caution. It is particularly unsatisfactory when applied to sharp intense resonances of residues at the termini of proteins. If the intensity of the peak at the final time point is substantial, the offset in a 3 parameter fit is poorly estimated (even when time points to a number of time constants have been measured). Unfortunately because of the high S/N of these decay curves the F-test will allocate a 3-parameter fit as being the best fit to the data.

Finally, it should be noted that since the effects of noise in the sampling of weak peaks is clearly of im-

Table 1. Comparison of 2 and 3 parameter fits to simulated data

Simulation parameters[a]			Mean (standard deviation) of 2 parameter fits		Mean (standard deviation) of 3 parameter fits		
I_0	I_∞	T[b]	I_o	R	I_o	R	I_∞
10	0	5	9.999 (0.038)	1.000 (0.006)	9.998 (0.037)	1.000 (0.010)	0.001 (0.029)
10	0	1	10.001 (0.031)	1.000 (0.007)	10.066 (0.267)	0.991 (0.041)	−0.070 (0.281)
1	0	5	0.999 (0.039)	0.999 (0.057)	1.000 (0.039)	0.998 (0.102)	−0.002 (0.029)
1	0	1	1.000 (0.032)	0.995 (0.068)	2.670 (7.096)	0.932 (0.442)	−1.672 (7.115)
10	0.2	5	10.108 (0.038)	0.935 (0.005)	9.999 (0.038)	1.000 (0.010)	0.201 (0.029)
10	0.2	1	10.186 (0.032)	0.969 (0.006)	10.051 (0.263)	0.993 (0.042)	0.147 (0.280)
1	0.2	5	1.076 (0.036)	0.506 (0.031)	1.002 (0.037)	1.000 (0.103)	0.198 (0.029)
1	0.2	1	1.191 (0.031)	0.762 (0.052)	2.067 (5.782)	0.945 (0.415)	−0.869 (5.796)

[a]For each combination of the three parameters, I_o, I_∞ and T, 1001 decay curves with 15 sample points and a rate of 1 s^{-1} were simulated. Normally distributed noise with a standard deviation of 0.05 was added to the curves. Curves were fitted with the program CurveFit (Palmer et al., 1991; Palmer, 1997).
[b]T = number of characteristic time constants sampled during the simulated relaxation decay.

portance in the evaluation of relaxation data for these resonances, it may be worthwhile simply to make use of the trade-off between the number of data points acquired and the reduction of noise that follows on from acquiring a larger number of transients per data point. For example, 1000 T_1 data points acquired at a given noise level N would have more serious problems in evaluation than 10 data points acquired at a noise level of N/10, which would take the same amount of spectrometer time.

Conclusions

We conclude that the following points can be followed to obtain the most accurate and precise relaxation rates. *Quantitation of peak intensity* should ideally be by the use of peak volumes unless there is resonance overlap present. If a search routine between successive spectra is used to obtain the peak height maximum, data that are sampled towards the end of the decay where the signal is comparable in intensity to the noise maximum should be excluded from the data analysis. *Fitting of relaxation decay curves* by the use of a 2-parameter fit to determine the rate is indicated in all cases unless there is a significant offset for a peak of low intensity. This paper has described some potential areas in which systematic bias may be introduced and suggested ways by which they can be avoided. We hope that this study will prove useful to others working on the analysis of relaxation dynamics in biopolymers.

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