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# Computation of relaxation matrix elements from incomplete NOESY data sets

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## SUMMARY

The structural determination of biological molecules in solution by NMR relies on the determination of a set of interatomic distances obtained by measurement of intramolecular nuclear Overhauser effects (NOE). It is shown in this paper that it is possible to obtain the accurate relaxation rate (and hence the interatomic distance) from the direct measurement of a single NOE signal. The precise analysis of a NOESY peak evolution with respect to the mixing time allows the evaluation of the relaxation parameters for the pair of spins under consideration. This is done without any assumption on the relaxation of unmeasured spins, or on the movement of the molecule. The theoretical basis of this method is presented. In order to evaluate the proposed method, a simulated case on the protein BPTI is studied, which shows that the method performs very well even in the case of noisy data sets.

## INTRODUCTION

NMR is an invaluable tool for the structural determination of biological molecules in solution. The method relies on the determination of a set of interatomic distances obtained from the molecule under study by the measurement of intramolecular nuclear Overhauser effects (NOE) (Macura and Ernst, 1980). Measurement of the NOE intensity provides an approximate value of the dipolar relaxation rate, from which the interatomic distances in the molecule can be evaluated. A 3D structure of the molecule can then be produced from the estimation of these distances (Crippen and Havel, 1978; Braun and Gō, 1985).

Several major problems arise from this approach. The first is that an accurate measurement of the NOE parameters does not lead to the derivation of accurate values for the interatomic distan-

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ces. This is due to the NOE parameter dependence on the value $J(\omega)$, the spectral density function of the movement of the molecule, which is usually unknown. A second problem is that, due to collective relaxation modes known as spin diffusion, it is very difficult to accurately evaluate the value of a relaxation parameter from the measurement of a single NOE intensity (Lane, 1988; Clore and Gronenborn, 1989). The complete interpretation of the relaxation pathways necessitates the handling of the relaxation matrix as a whole (Borgias and James, 1988). One way of circumventing this problem is to measure the values of the NOE intensities at very short mixing times for which spin diffusion has not yet occurred (Kumar et al., 1981). This approach however, is only rigorously valid for infinitely small NOE intensities, and thus remains biased for realistic measurements. A better approach is to measure as many NOEs as possible, and to reconstruct the complete relaxation matrix from this (usually) partial set of values. Several methods (Boelens et al., 1988; Borgias and James, 1989; Koehl and Lefèvre, 1990; Gippert et al., 1990; Madrid et al., 1991; Baleja et al., 1990; Mertz et al., 1991; Bonvin et al., 1991) have been proposed to overcome the problem of an incomplete set of values, but the number of quantified NOE peaks always influences the accuracy of the final result. Some of these methods rely on some kind of molecular modelling to compensate for the lack of measured NOEs and, as such, depend on a model for the movement of the molecule.

In this paper we propose a method for obtaining accurate relaxation rates from the direct measurement of a single NOE signal. This method relies on a precise analysis of the NOE peak evolution with respect to the mixing time in a NOESY experiment. This analysis allows the evaluation of the relaxation parameters for the pair of spins under consideration, without extra assumptions on the relaxation of unmeasured spins, or on the movement of the molecule. We will first describe the theoretical basis of this method, and follow this with a simulated case on the protein BPTI, in order to evaluate the proposed method.

## THEORY

One can express the NOE intensities obtained from a NOESY experiment as a series of symmetric matrices $I(\tau_m)$, where $I_{ij}(\tau_m)$ holds the intensity of the cross peak between atoms i and j, for a NOESY mixing time of $\tau_m$. The relaxation parameters for each pair of spins can be presented as a matrix (the relaxation matrix) $\Sigma$. $\Sigma$ is connected to the NOE intensity matrix $I(\tau_m)$ by the relation (Keepers and James, 1984):

$$I(\tau_m) = \exp(-\Sigma \tau_m) I_o \qquad (1)$$

where $I_o$ is a diagonal matrix with elements equal to the equilibrium magnetizations of each respective spin.

If the complete experimental determination of the $I(\tau_m)$ matrix can be performed at a given $\tau_m$, then the determination of $\Sigma$ is straightforward (Olejniczak et al., 1986). However, this is seldom the case, due to overlap in the 2D NOESY spectra.

The problem is then one of incompleteness of $I(\tau_m)$ and several procedures have been proposed to interpret quantitative NOESY information in terms of the relaxation matrix $\Sigma$. We will show here, that this incompleteness can be avoided if a different approach to the problem is taken.

The relaxation matrix is symmetric, provided we use the construction proposed by Olejniczak

(1989). It can be diagonalized:

$$\Sigma = L \Lambda L^{t} \tag{2}$$

where $\Lambda$ is a diagonal matrix, and I is thus computed by:

$$I = L \exp(-\Lambda \tau_m) L^{t} \tag{3}$$

From Eq. 2 we can rewrite each element of matrix $\Sigma$ in the following way:

$$\Sigma_{ij} = \sum_k L_{ik} L_{jk} \lambda_k \tag{4}$$

so that each element of matrix I becomes:

$$I_{ij} = \sum_k L_{ik} L_{jk} \exp(-\lambda_k \tau_m) \tag{5}$$

where the $L_{ik}$ is the (i,k) element of the matrix L, and $\lambda_k$ is the $k^{th}$ element of the diagonal matrix $\Lambda$. We can rewrite Eqs. 4 and 5 as follows:

$$I_{ij}(\tau_m) = \sum_k A_{ij}^k \exp(-\lambda_k \tau_m) \tag{6}$$

and:

$$\Sigma_{ij} = \sum_k A_{ij}^k \lambda_k \tag{7}$$

where:

$$A_{ij}^k = L_{ik} L_{jk} \tag{8}$$

The form of Eq. 6 proves that NOESY build-up curves are sums of exponentials. Equations 6 and 7 show that we can deduce the value of the relaxation matrix elements from the analytical form of the build-up curve.

The process we propose for computing the relaxation matrix elements from the NOESY experimental intensities is then, given an experimental NOE build-up curve $I_{ij}(\tau_m)$ measured between atoms i and j, to extract the parameters $A_{ij}^k$ and $\lambda_{ij}^k$ of the multi-exponential decay. Because of the bad accuracy of experimental build-up curves, no effort will be made to correlate the $\lambda_{ij}^k$ values for the different {i,j} atom pairs under consideration.

From the parameters $A_{ij}^k$ and $\lambda_{ij}^k$ it is then easy to obtain the values of the relaxation parameter $\Sigma_{ij}$. Subsequently, it is possible to extract distance information by assuming a dynamic model for the molecule (for instance rigid spherical tumbling).

It is well known that extracting parameters from a sum of exponentials is a major challenge in

numerical analysis. However, the problem is eased here by the fact that we have:

$$\sum_k A_{ij}^k = 0 \tag{9}$$

as the cross-relaxation intensity at $\tau_m = 0$ is null.

The LP-SVD method (Barkhuijsen et al., 1985) has been chosen in this work, because it permits the separation of the signal parameters from additional parameters arising from the noise (Kumaresan and Tufts, 1982).

## MATERIALS AND METHODS

To test and exemplify the method, we chose to analyze a set of build-up curves simulated from BPTI, a small protein of 57 residues, the crystallographic structure of which, taken from the Protein Data Bank (6PTI), has been determined at 1.7 Å (Wlodawer et al., 1987).

The hydrogen atoms, absent in the X-ray structure, were added in a straightforward manner, using the DISCOVER program (Biosym Technologies). The structure was then energy-minimized to remove any remaining bad steric contacts.

The program CORMA (Keepers and James, 1984) was used to simulate build-up curves at various $\tau_m$ from the molecular structure. With $\tau_m$ being varied from 0 to 2.0 s, three data sets were produced consisting of 30, 40 and 50 points, sampling the build-up curve at regular intervals of respectively 67 ms, 50 ms and 40 ms.

The LP-SVD method (Barkhuijsen et al., 1985) was used to extract the exponential parameters from the build-up curves, according to Eq. 6. The LP-SVD method is based on a Singular Value Decomposition analysis of the data. From this analysis, a polynomial is constructed (the so-called autoregressive backward polynomial) from which the damping factors of the exponentials present in the signal are evaluated. The autoregressive backward polynomial has the property that all the complex roots located outside the unit-circle (the signal-related roots) are related to damped sinusoids present in the signal, whereas the roots located within the unit-circle are related to noise and do not bear any relevant information (Kumaresan and Tufts, 1982). The frequencies and the damping factors of each sinusoid found in the signal are then extracted from the signal-related roots as being, respectively, the phase and the inverse of the amplitude of each root. For the analysis of build-up curves, only real roots should be found outside the unit-circle, since no frequencies are present in the analyzed signal. From the evaluation of the damping factors of exponentials present in the signal, a simple least-squares analysis then permits the extraction of the amplitudes related to each exponential.

When the noise level is high, however, instability in the polynomial rooting may generate complex roots outside the unit-circle. In this case, these roots are found as complex conjugated doublets called 'Froissard doublets' (Aubard et al., 1987). Such doublets lead to inaccurate reconstruction of the respective relaxation matrix elements. To circumvent this problem, we propose the rotation of such doublets about their center, before the amplitude estimate, in order to bring the roots back to the real axis. It is shown in the appendix that this operation does not modify the first order of the build-up curve reconstruction and can be safely used when the imaginary part of the complex root is small compared to the real part. This condition can be translated into geometric terms, by enforcing that the complex roots must lie within a cone of angular extent $\alpha$ (Fig. 1).

In some cases however, particularly when the signal-to-noise ratio is very low, the LP-SVD analysis produces complex root doublets corresponding to higher frequencies for which the condition of Fig. 1 no longer holds. In this case the fast frequencies detected in the signal are probably associated to the noise. We thus chose to ignore the related roots altogether for the amplitude reconstruction step. This decision was supported by the fact that the corresponding amplitudes, when computed, often appear to be at least one order of magnitude smaller than the other amplitudes.

From the multiexponential analysis performed on the build-up curve as described, the relaxation parameter $\Sigma_{ij}$ is computed as shown in Eq. 7. It is obvious that the accuracy of the determination of the relaxation parameter is critically related to the accuracy of the multiexponential analysis. In order to check the quality of this analysis, we computed an 'R factor' much in the way X-ray crystallographers do.

$$R = \frac{\sum_k |x_k^{calc} - x_k^{exp}|}{\sum_k |x_k^{exp}|} \tag{10}$$

Here $x_k^{calc}$ is the value of the calculated build-up curve reconstructed from the multiexponential parameters, and $x_k^{exp}$ is the 'experimental' data, for k running on the different values of $\tau_m$. The R factors were computed for each spin pair studied since each pair is independent. We found the R factor to be more discriminant when computed only for small values of $\tau_m$.

The method described here is essentially designed to calculate the relaxation rates of proton pairs. But, to check its validity in a more sensitive manner, we decided to calculate the interproton distances from the relaxation rates, in order to compare them to those extracted directly from the crystallographic structure.

From the rates, the distances were calculated using a classic method: having chosen a calibration rate $\Sigma_o$, corresponding to the known distance $d_o$, all other distances $d_{ij}$ were obtained from the rates $\Sigma_{ij}$:

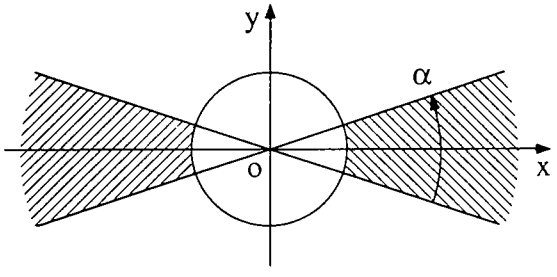


Fig. 1. Visualization of the position of complex roots in the complex plane. When the imaginary part of the roots is small with respect to their real parts, the roots are in a cone of angular extent α. The angular extent depends on the ratio of imaginary and real parts. To apply root pivoting, we choose a ratio less than one-sixth, implying an angle of less than approximately 15°. Complex root doublets appearing in the shaded area are corrected by the root-pivoting operation (see text). Complex root doublets appearing outside this area are discarded.

the rates $\Sigma_{ij}$:

$$\frac{\Sigma_o}{\Sigma_{ij}} = \left(\frac{d_{ij}}{d_o}\right)^6 \tag{11}$$

The calibration distance chosen was the 1.74 Å distance of the αH pair of $Gly^{56}$.

For the stability study, the processing was also performed for increasing levels of random noise present in the analyzed data set. The added noise had a Gaussian distribution and was generated with a congruent algorithm.

All the methods described here were written in Fortran and implemented in the GIFA (Delsuc, 1989) NMR data-processing program developed in our laboratory and available from the authors. The parameter extraction by the LP method was performed on an Alliant VFX 40 computer.

A typical build-up curve and the result of the analysis are shown in Fig. 2.

## RESULTS

From the NOE simulation of BPTI, we selected all the proton pairs giving NOEs greater than 0.5% at a 200-ms mixing time, and which had interatomic distances shorter than 6.0 Å, for a total of 2304 proton pairs. Of the simulated data set, 55% of the simulated build-up curves had a mean intensity value equal to or smaller than 5% intensity in NOE units (defined here as the ratio of the peak to the diagonal peak of a single hydrogen for $\tau_m = 0$).

A first test was prepared from the 50-points data sets, with no added noise. Out of the 2304 build-up curves, 1352 were analyzed without difficulty, 785 needed the application of pivoting as described above, 200 were found with roots with large imaginary parts (the roots of which were thus rejected), 1 curve could not be analyzed because it led to a positive relaxation element, and 2 curves had R factors greater than 0.25 and were thus rejected. The results for the 2301 determined distances compared to the crystallographic distances are presented in Fig. 3a. The result of

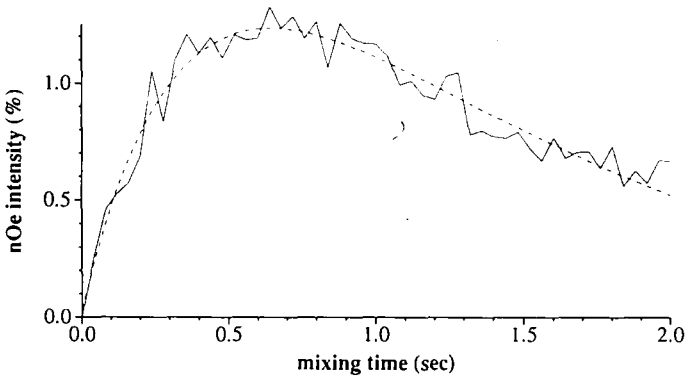


Fig. 2. Comparison between simulated (solid line) curve and results of the analysis (dashed line), for the αH ($Arg^1$)–β2H ($Cys^{55}$) pair in BPTI. The theoretical distance is 3.72 Å, the computed one 3.76 Å. The noise level is 0.1%. The R factor is 0.24.

a more classical 'initial slope' analysis performed on the same data set is shown in Fig. 4, using the first 100-ms data points.

Subsequently, three other tests were prepared from the 50-points data sets with added noise at levels of 0.02%, 0.1% and 0.5%, in NOE units.

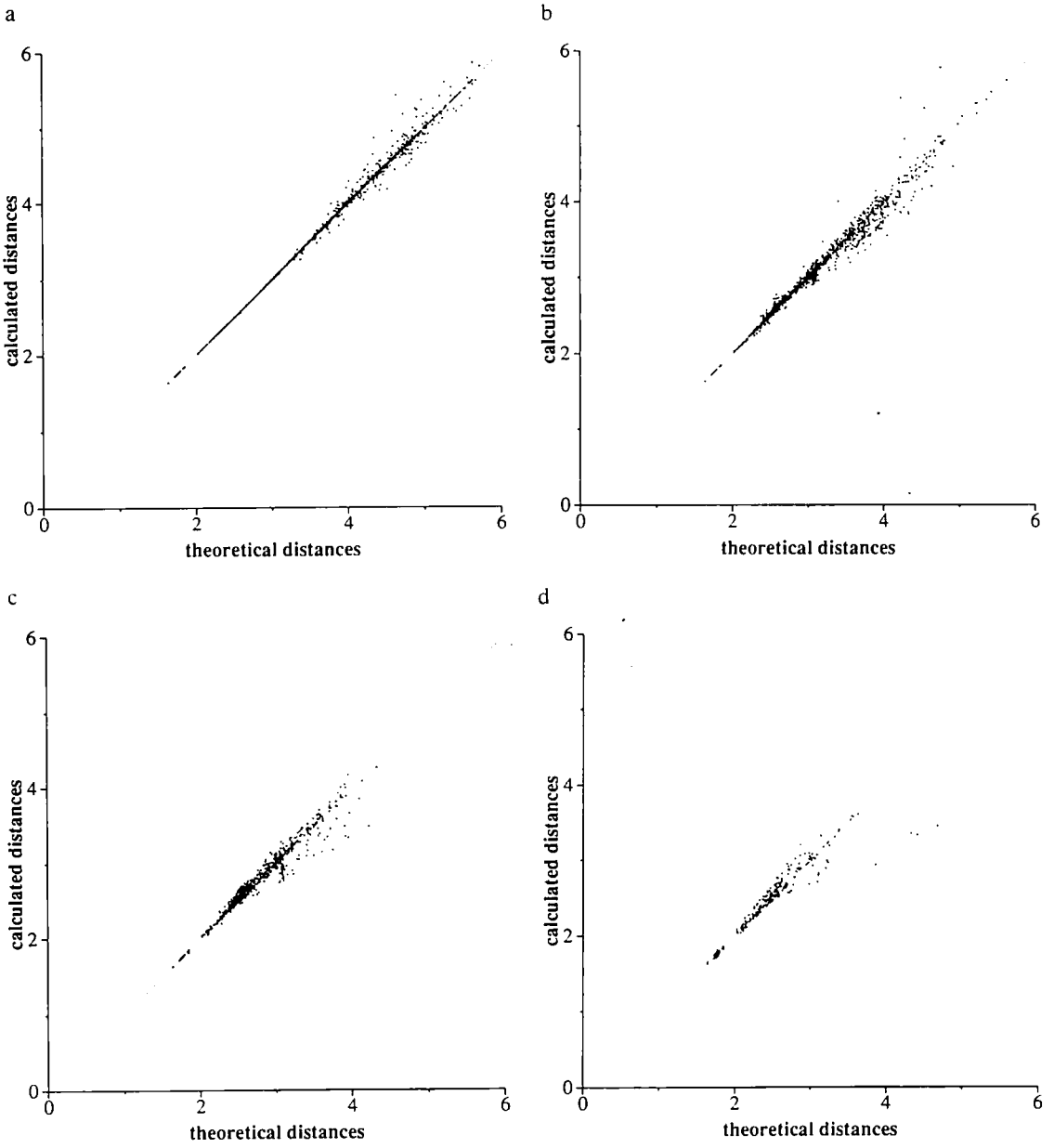


Fig. 3. Plot of the calculated distances by the LP-SVD method (y-axis) as a function of theoretical distances (x-axis) on the 50-points data set, with different noise levels: 0% (a), 0.02% (b), 0.1% (c) and 0.5% (d). In each plot, proton pairs giving an R factor greater than 0.25 were removed; distances are in Å.

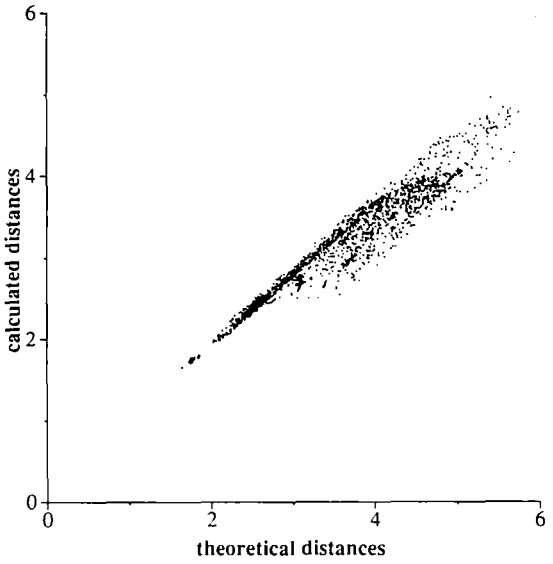


Fig. 4. Plot of the calculated distances by the initial-slope method (y-axis) as a function of theoretical distances (x-axis) without noise. Distances are in Å. The slope was obtained from the two points at $\tau_m$ = 50 ms and $\tau_m$ = 100 ms, assuming a two-spin relaxation mechanism.

For the first data set corresponding to a noise level of 0.02%, 1062 curves were analyzed without further processing, 955 required the use of pivoting, 387 were found with roots with large imaginary parts (roots were thus rejected), 37 curves could not be analyzed as they led to positive relaxation elements, 7 curves had no real roots, and 961 curves had R factors greater than 0.25 and were thus rejected. The results for the 1299 determined distances compared to the crystallographic distances are presented in Fig. 3b.

For the noise level of 0.1%, 1088 curves were directly analyzed, 931 required pivoting, 315 were found with roots with large imaginary parts (roots were rejected), 27 curves could not be analyzed as they led to positive relaxation elements, 70 curves had no real roots, and 1325 curves had R factors greater than 0.25 and were thus rejected. The results for the 882 determined distances compared to the crystallographic distances are shown in Fig. 3c.

For the third noise level of 0.5%, 679 curves were directly analyzed, 752 required pivoting, 194 were found with roots with large imaginary parts (roots rejected), 39 curves could not be analyzed as they led to positive relaxation elements, 738 curves had no real roots, and 1184 curves had R factors greater than 0.25 and were thus rejected. The results for the 343 determined distances compared to the crystallographic distances are shown in Fig. 3d.

The quality of the analyses was then evaluated by computing the error distribution using the quantity $\Delta_i = \frac{d_i^{calc}}{d_i^{theo}}$, computed for all the distances determined.

The mean, $<\Delta>$, and the standard deviation, $\sigma$, were then computed for each noise level. A departure of $<\Delta>$ from unity is a sign of a bias in the method, and a small $\sigma$ value parameter indicates a small overall error in the determined distances. These results are summarized in Table 1.

TABLE 1
SUMMARY OF NUMERICAL TESTS FOR THE QUALITATIVE EVALUATION OF THE ANALYSIS OF BUILD-UP CURVES

| Noise level (%) | $<\Delta>$ | $\sigma$ (%) | Maximum distance found (Å) | Number of distances kept |
|---|---|---|---|---|
| 0 (sl)[a] | 0.89332 | 6.19 | 5.73 | 2304 |
| 0 (lp)[a] | 1.00009 | 0.98 | 5.73 | 2301 |
| 0.02 (lp)[a] | 0.99761 | 2.54 | 5.61 | 1299 |
| 0.1 (lp)[a] | 1.00559 | 2.83 | 5.14 | 882 |
| 0.5 (lp)[a] | 1.00492 | 3.89 | 4.67 | 343 |

[a] lp, linear prediction; sl, slope method. When the linear-prediction method is used, only the points giving an R factor less than 0.25 are considered in the analysis.

The same analyses were then performed on the 40-points and 30-points data sets, with the same kind of results. A summary of the quality of the analysis is shown in Table 2.

## DISCUSSION

The method described here permits quantitative estimations of interproton distances in a protein from the analysis of NOE build-up curves. It has several advantages over the procedures previously proposed.

First, it is essentially a *local* method; the determination of the interproton distance i,j is performed only from the build-up curve measured between spins i and j. The accuracy of the obtained parameter is totally independent of other measurements, and the analysis can be performed on an isolated peak, for which the assignment need not even be known. This is in sharp contrast with all the other methods developed for this purpose. With the exception of the initial-slope method, all the proposed techniques for the determination of distances from the NOE intensity measurement rely on some kind of back-calculation of the NOE intensities from a set of geometrical parameters. These parameters can be either a rough 3D molecular structure (Borgias and

TABLE 2
QUALITATIVE EVALUATION OF THE ANALYSIS OF BUILD-UP CURVES FOR DIFFERENT SAMPLINGS

| Noise level (%) | | 0 | 0.02 | 0.1 | 0.5 |
|---|---|---|---|---|---|
| 30 points | $<\Delta>$ | 1.00006 | 0.99855 | 1.00173 | 1.00137 |
| | $\sigma$ (%) | 2.02 | 3.31 | 4.21 | 5.49 |
| 40 points | $<\Delta>$ | 0.99991 | 1.0003 | 1.0014 | 1.01418 |
| | $\sigma$ (%) | 0.78 | 2.84 | 3.19 | 4.17 |
| 50 points | $<\Delta>$ | 1.00009 | 0.99761 | 1.00559 | 1.00492 |
| | $\sigma$ (%) | 0.98 | 2.54 | 2.83 | 3.89 |

James, 1989; Boelens et al., 1988) or a set of distances (Madrid et al., 1991). In all cases, unmeasured NOE peaks are estimated from the current estimate of the geometrical parameters. All these techniques are characterized by the fact that the number of unmeasured NOE peaks must not be too large for the method to converge, and also by the fact that a large number of unmeasured NOE peaks will impair the precision of the distance determinations.

It should also be noted that the method is 'model-free'. Indeed, the purpose of the computation is to obtain relaxation parameters and not distances. The user can always infer the distances from the relaxation parameters by choosing an adequate dynamic model for the molecule. The efficiency of the method is completely independent of the choice of this model.

The analysis of the results shown in Figs. 3 and 4 and in Tables 1 and 2 proves that the proposed method performances are very good. In the ideal case of a noise-free data set, the method performs ideally, with no error of any kind for distances up to 6 Å. Despite the fact that this case is unrealistic, it shows how intrinsically suitable the method is. The results shown in Fig. 3b–d demonstrate the method's performance on more realistic noisy data. The evaluation of the R factors following Eq. 10 leads to the rejection of build-up curves that can not be efficiently analyzed. As a result, as indicated in Table 1, increasing levels of experimental noise do not increase significantly the errors on the computed distances, but only reduce the number of distances that can be successfully extracted. This feature ensures that the measures obtained will maintain a constant level of confidence, determined by the threshold chosen for rejecting build-up curves with large R values, regardless of the noise level. For the value R = 25%, chosen here, the error on the obtained distance is in the order of 4%.

The method seems to perform equally well with different samplings of the build-up curves. The results shown in Table 2 are actually biased by the fact that the R factors were computed at different positions (because of the different sampling periods); this results in a slightly less tight selection of the incorrect distances leading to slightly larger values for $\sigma$.

Although LP-SVD is one of the slowest linear-prediction methods, it is very efficient on small data sets, thus the method proposed here performs relatively quickly. The complete analysis of the 2304 curves was performed in about 90 min on our Alliant FX 40 with a mean processing time of 2.3 s/curve.

The application of these techniques to experimental studies may now be investigated. Several problems arise when an experimental approach is attempted.

First, it may seem unrealistic to perform 40 or 50 NOESY experiments on a typical sample of protein. However, the development of 3D experiments has shown that good-quality NOESY spectra can be acquired in a couple of hours on protein samples with sufficient sensitivity. Thus, acquiring such a set of NOESY experiments would require 3 to 4 days, which seems reasonable given the workload of a typical NMR spectrometer.

Second, it appears from the simulation of the build-up curves that very different curve shapes are present in the experiment. Some reach their maxima at very short mixing times and then decay rapidly, while others increase slowly, attaining their maxima at long mixing times. Thus, one should carefully set up the sampling of the mixing time delay so that an optimal accuracy is obtained for relaxation-rate estimation.

Another limitation of the method in the present state of its implementation is that linear prediction needs to run on regularly sampled points. This leads to a nonoptimal measurement of the build-up curves in their decaying part, where the density of information is low. This shortcoming

may be surmounted by using other analytical methods, such as the method based on the Padé–Laplace analysis (Yeramian and Claverie, 1987).

Another characteristic of the experimental data set will be the presence of zero-quantum peaks in the NOESY spectra. These signals will produce a sinusoidal modulation of the measured peak with respect to $\tau_m$, added to the NOE modulation. The analysis of a build-up curve containing frequencies due to zero-quantum peaks may be even more difficult. However, we do not expect this effect to be a major burden as: (i) the lifetime of the zero-quantum coherences for large molecules seems to be at least an order of magnitude smaller than the $T_1$ and NOE lifetimes; and (ii) since the zero-quantum peaks have an antiphase pattern, with zero volume, a careful integration of the peak pattern should cancel this effect (Stoven et al., 1989).

## CONCLUSION

The method presented here should be a valuable tool for the determination of interproton distances in biological molecules. It gives encouraging results on simulated studies, but experimental cases remain to be tested. It should permit the extraction of quantitative distance information from NMR studies. Accuracy better than 5% for distances up to 4–5 Å (as seen in Fig. 3) does not seem unrealistic. Such an accuracy, if verified experimentally, would change the approach of structural determination of biological molecules by NMR. Structural information could be gained from a small set of accurate distances, for instance in cases where assignment is not fully available, rather than on a large set of inaccurate distances as is commonly practised at present.

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## APPENDIX

We show here that when Froissard doublets are obtained from a rooting analysis of the autoregressive polynomial, the rotation of such a doublet by $\frac{\pi}{2}$ in order to bring the roots back to the real axis gives rise to equivalent build-up curve patterns. To do so, we show that the curves thus obtained have equivalent first-order estimation.

A Froissard doublet is characterized by two related roots, located within the unit-circle, with opposite frequencies, opposite imaginary phases and the same amplitudes and damping factors:

$$z_1 = e^{a+ib} \qquad z_2 = e^{a-ib} \tag{A1}$$

The time dependence generated by such a pattern is:

$$-i\,e^{(a+ib)t} + i\,e^{(a-ib)t} = i\,e^{at}(e^{-i\,bt} - e^{i\,bt}) = 2e^{at}\sin(bt) \sim 2\,bt\,e^{at} \quad \text{if } bt \ll 1 \tag{A2}$$

The rotation leads to:

$$z'_1 = e^{a+b} \qquad z'_2 = e^{a-b} \tag{A3}$$

which gives:

$$e^{(a+b)t} - e^{(a-b)t} = e^{(a+b)t}(1 - e^{-2\,bt}) \sim 2\,bt\,e^{at} \quad \text{if } bt \ll 1 \text{ and } b \ll a \tag{A4}$$

The developments of the two expressions are very similar and equivalent for small values of bt. The operation permitting the replacement of Eq. A2 by Eq. A4 is equivalent to pivoting the root doublet by $\frac{\pi}{2}$ about the center of the doublet, bringing the roots back onto the real axis.