Interpretation and Analysis of ^{15}N Relaxation Data

Anisotropic rotational diffusion and internal mobility using the Lipari-Szabo Approach using the program TENSOR2

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Introduction

What's New in Tensor Version 2.0?

Firstly and most importantly, Tensor now carries out an analysis of internal motion using the Lipari-Szabo or extended Lipari-Szabo type analysis. It's about time. This can either be performed using an isotropic rotor to describe rotational diffusion, or using the anisotropic tensor determined using a subset of spins, themselves assumed to have a negligible component to their internal motion. The internal mobility analysis is performed using the logic introduced by the NIH lab (Clore et al 1990),and reproduced by Professor Art Palmer's lab (Mandel et al 1995), sequentially fitting with more complicated models until the data are adequately reproduced. The confidence limits and uncertainty in the parameters are determined using a rapid monte-carlo based statistical analysis. As has been noted by numerous authors, the error function defined by the fitted internal mobility parameters $\{S^2,ti\}$ is highly non-linear when fitting R1,R2 and heteronuclear nOe. It is now possible to rapidly scan the monte-carlo simulations from the fit of each ^{15}N site to check the definition of the optimised internal mobility parameters.

The program also has undergone a large number of cosmetic improvements (ribbon and ball-and-stick display options) and the local motion can now be indicated using colour scales on the molecule. Interactive residue-identification using the cursor aids in recognising more or less mobile parts of the molecule.

Overall rotational diffusion.

The time autocorrelation function is affected by both internal and overall motion, implying that both contributions require detailed attention, if the motion of the interactions, and consequently the relaxation, are to be adequately described.

In the simplest analysis, overall motion is assumed to be isotropic and this contribution is described by a single exponential.

In the case of rotational diffusion anisotropy the time correlation is described by a multiexponential function whose relative weighting depends on the orientation of the different relaxation interactions with respect to the principal components of the diffusion tensor.

Not surprisingly the interpretation of heteronuclear relaxation using the model-free approach assuming isotropic motion can artificially evoke fictive contributions to the internal motion if the rotational diffusion tensor is actually significantly anisotropic (Schurr et al 1994).

In order to interpret the internal motion with confidence, an accurate description of the contribution to the time correlation function due to overall motion is therefore necessary. It has recently been shown that this tensor can be accurately determined from the relaxation rate ratio R_2/R_1, which becomes independent of internal motion in the fast motion limit and is highly sensitive to overall diffusion.

In order to reduce the possibility of contributions from internal motion in this analysis it is important to select residues whose interaction directions are likely to be in the librational motional limit. This is achieved either by analysis of the actual relaxation rates with repect to the mean values for the whole molecule, or by identifying those residues present in secondary structural elements which are assumed to be similarly rigid. Once the rotational diffusion tensor has been determined using a subset of the available residues, this can be applied to a model-free analysis of the entire molecule.

TENSOR2 allows the determination of the rotational diffusion tensor from three-dimensional structure coordinates and ^{15}N relaxation data. Confidence in the various models available to describe the tensor is estimated using Monte-Carlo sampling methods in combination with appropriate $\chi^2$and F-tests, made possible by a highly efficient simulated annealing algorithm.

A graphical interface allows the visualisation of the orientation of tensorial components with

respect to the three-dimensional coordinates of the molecule, and provides a graphical presentation of the statistical behaviour of the system.

Internal Mobility

You can then use this tensor, or an isotropic model, to describe rotational diffusion in a Lipari-Szabo type analysis of internal motion.

Theory

Relaxation Data Analysis.

The ^{15}N heteronuclear relaxation rates R_1 and R_2 depend on the spectral density function $J(\omega)$, in the following manner

$$R_1 = d^2[J(\omega_{H\,-}\,\omega_N) + 3J(\omega_N) + 6J(\omega_{H+}\,\omega_N)] + c2J(\omega_N)$$

$$R2 = (d^2/2)[4J(0) + J(\omega_{H\,-}\,\omega_N) + 3J(\omega_N) + 6J(\omega_{H\,+}\,\omega_N) + 6J(\omega_H)] + (c^2/6)[4J(0) + J(\omega_N)]$$

$$\text{where } d^2 = (1/10)\,\gamma_H 2/\gamma_N 2(h/2\pi\iota)^2\cdot(r_{NH}^{-3})^2$$

$$\text{and } c^2 = (2/15)\,\omega_N{}^2\,(\sigma_{\|\,-}\,\sigma_\perp\,)^2$$

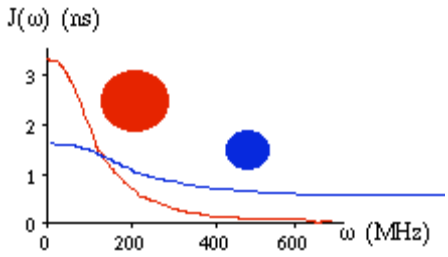
h is Planck's constant, $\gamma_H$and $\gamma_N$the gyromagnetic ratios of 1H and 15N, ω_H and ω_N their Larmor frequencies, and r_{NH} the internuclear distance (assumed to average to 1.01Å). $\sigma_{\|\,-}\,\sigma_\perp$ are the parallel and perpendicular components of the axially symmetric ^{15}N chemical shift tensor (approximated to -170ppm) which in a first approximation is assumed to be coaxial with respect to the dipolar interaction.

Isotropic tumbling

In the case of isotropic tumbling of the molecule, and rapid internal mobility ($\tau i < \tau c$) which is negligible in terms of relaxation effects, the auto-correlation function is described by a single decaying exponential and the spectral density function is given by:

$$J(\omega) = \frac{2}{5}\frac{S^2\tau_c}{1+\omega^2\tau_c^2}$$

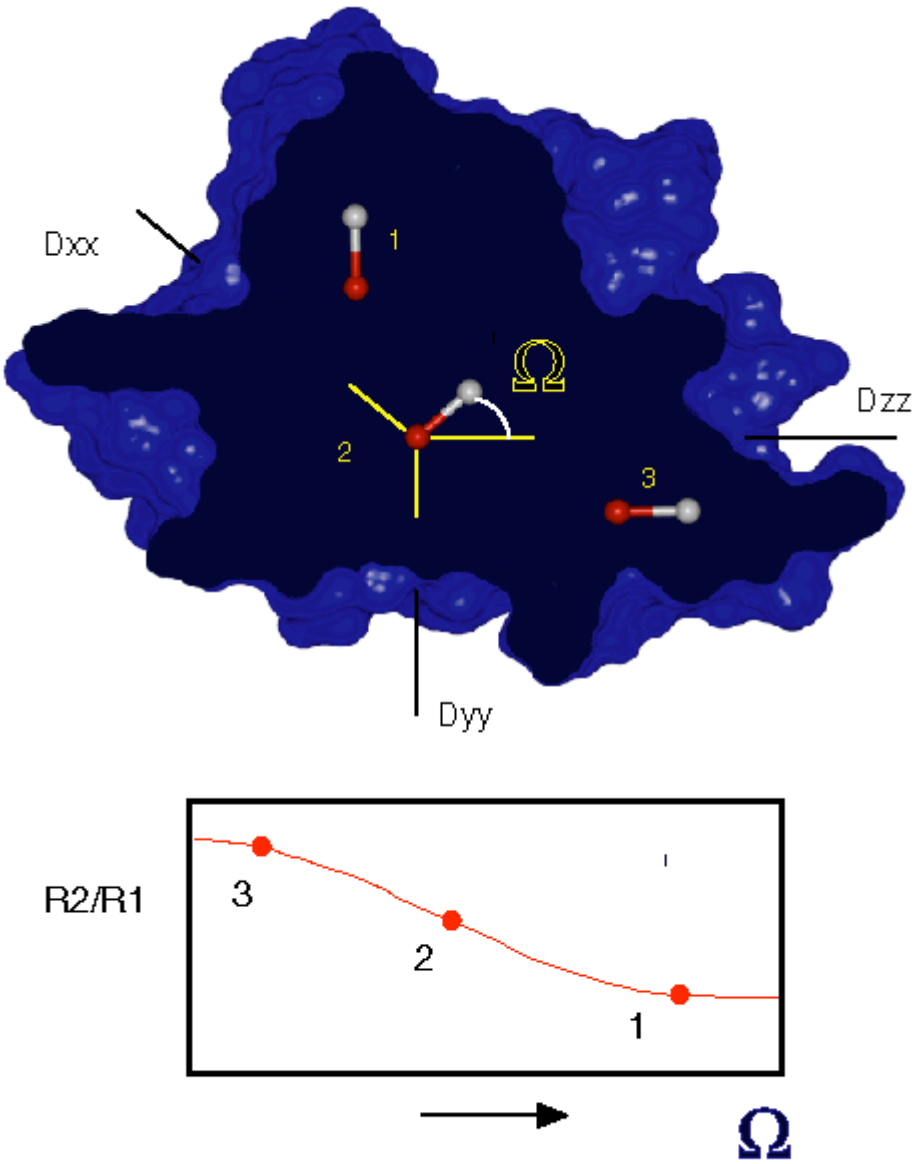
where τ_c is the overall correlation time of the molecule.



As long as we are careful to exclude residues whose internal mobility is negligible, the overall correlation time of the molecule can be fitted to the ratio R_2/R_1 which in this motional regime, is determined by τ_c.

Anisotropic tumbling: Fitting of the anisotropic rotational diffusion tensor.

In the case of anisotropic tumbling, the relaxation rates are dependant on the orientation of the relaxation mechanisms, relative to the rotational diffusion tensor of the molecule.



The spectral density function describing anisotropic rotational diffusion (Woessner 1962) is given by:

$$J(\omega) = \sum_i \left\{ A_i \tau_i / 1 + (\omega \tau_i)^2 \right\}$$

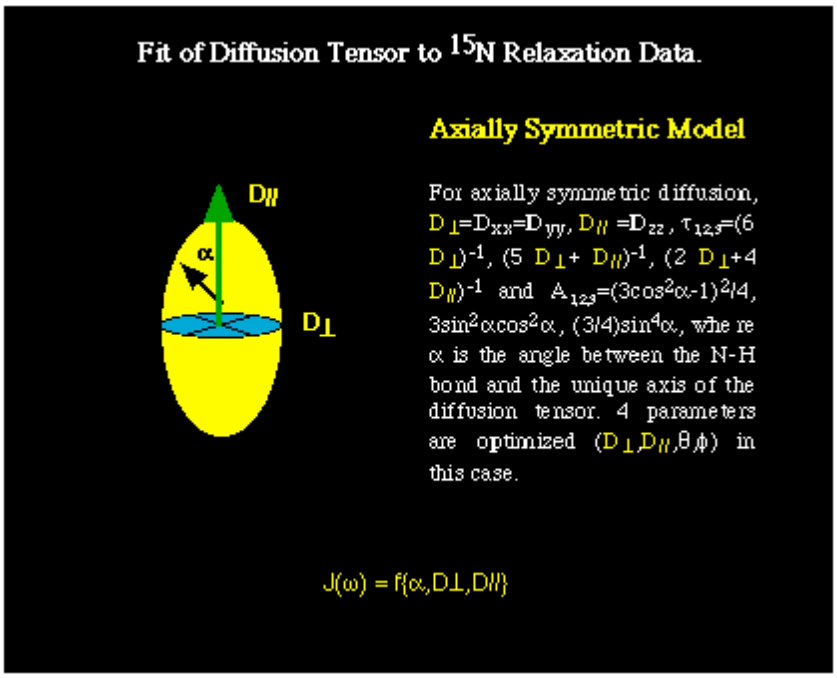
where $A_1=3y^2z^2$, $A_2=3x^2z^2$, $A_3=3x^2y^2$,
$A_{4,5}=0.25\{3(x^4+y^4+z^4)-1\}\pm(1/12)\{\delta_x((3x^4+6y^2z^2-1)+\delta_y((3y^4+6x^2z^2-1)+\delta_z((3z^4+6y^2x^2-1)\}$
and $\tau_{1,2,3}=(4Dxx+Dyy+Dzz)^{-1},(D_{xx}+4D_{yy}+D_{zz})^{-1}$, $(D_{xx}+D_{yy}+4D_{zz})^{-1}$,
$\tau_{4,5}=(6Diso\pm6(Diso2-L2)^{1/2})$, $Diso=(Dxx+Dyy+Dzz)/3$,

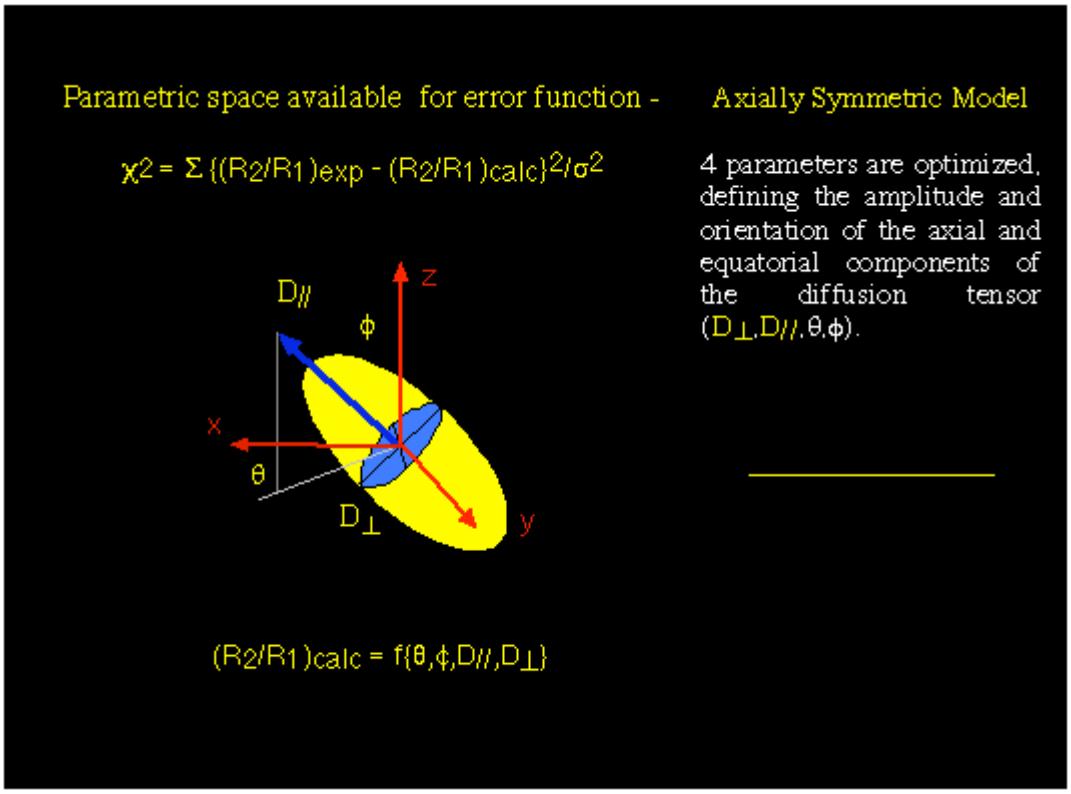
L^2=(DxxDyy+DxxDzz+DyyDzz)/3 and δ_m=(Dmm-Diso)/($Diso^2$-L^2) $^{1/2}$. m=(x,y,z) and (x,y,z) are the direction cosines of the N-H vector in the principal axis frame of the diffusion tensor.

6 parameters (D_{xx},D_{yy},D_{zz},alpha,beta,gamma) are optimized, describing the orientation and amplitude of the principle components of the diffusion tensor in the chosen molecular frame. The diffusion parameters are extracted by minimizing:

$$\chi^2 = \sum_i \left\{ \left[\left(R_2^{meas} / R_1^{meas} \right) - \left(R_2^{calc} / R_1^{calc} \right) \right]_i / \sigma_i \right\}^2$$

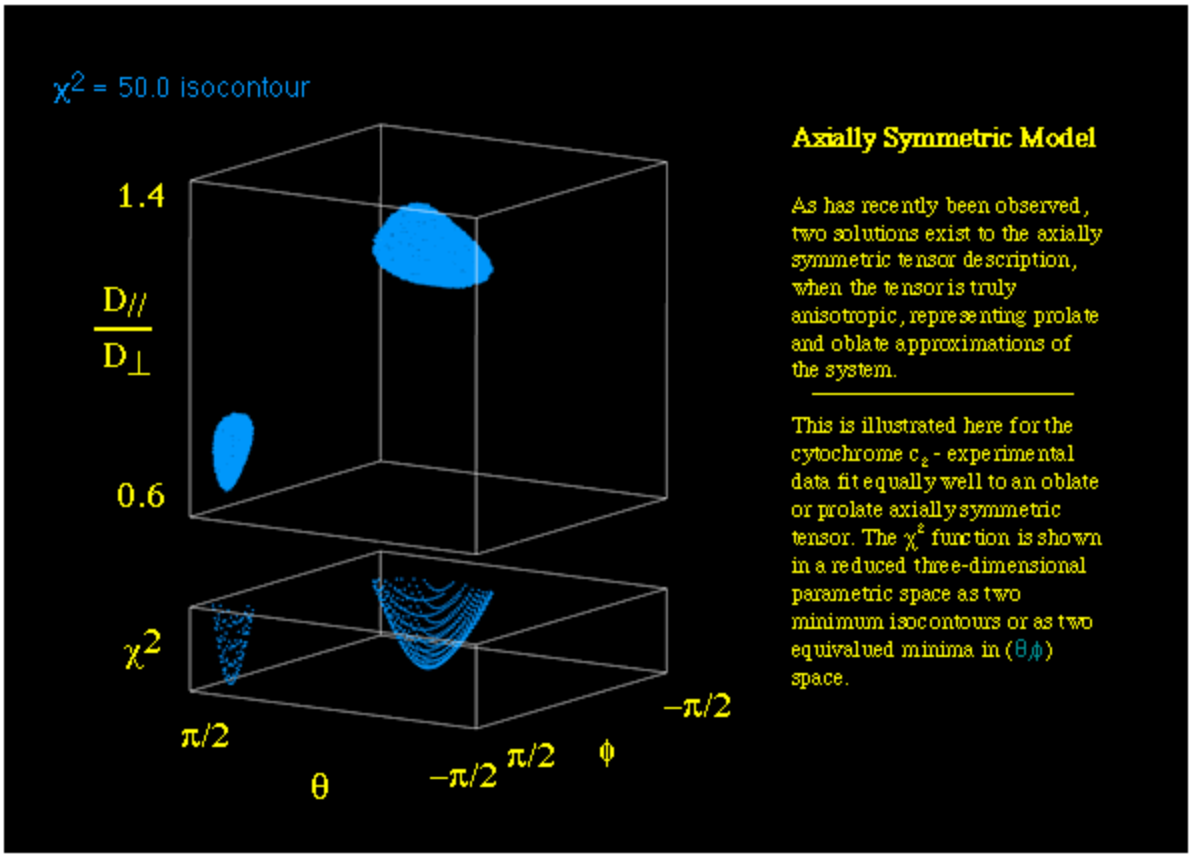
where σ is the uncertainty in the experimental R_2/R_1 ratio.



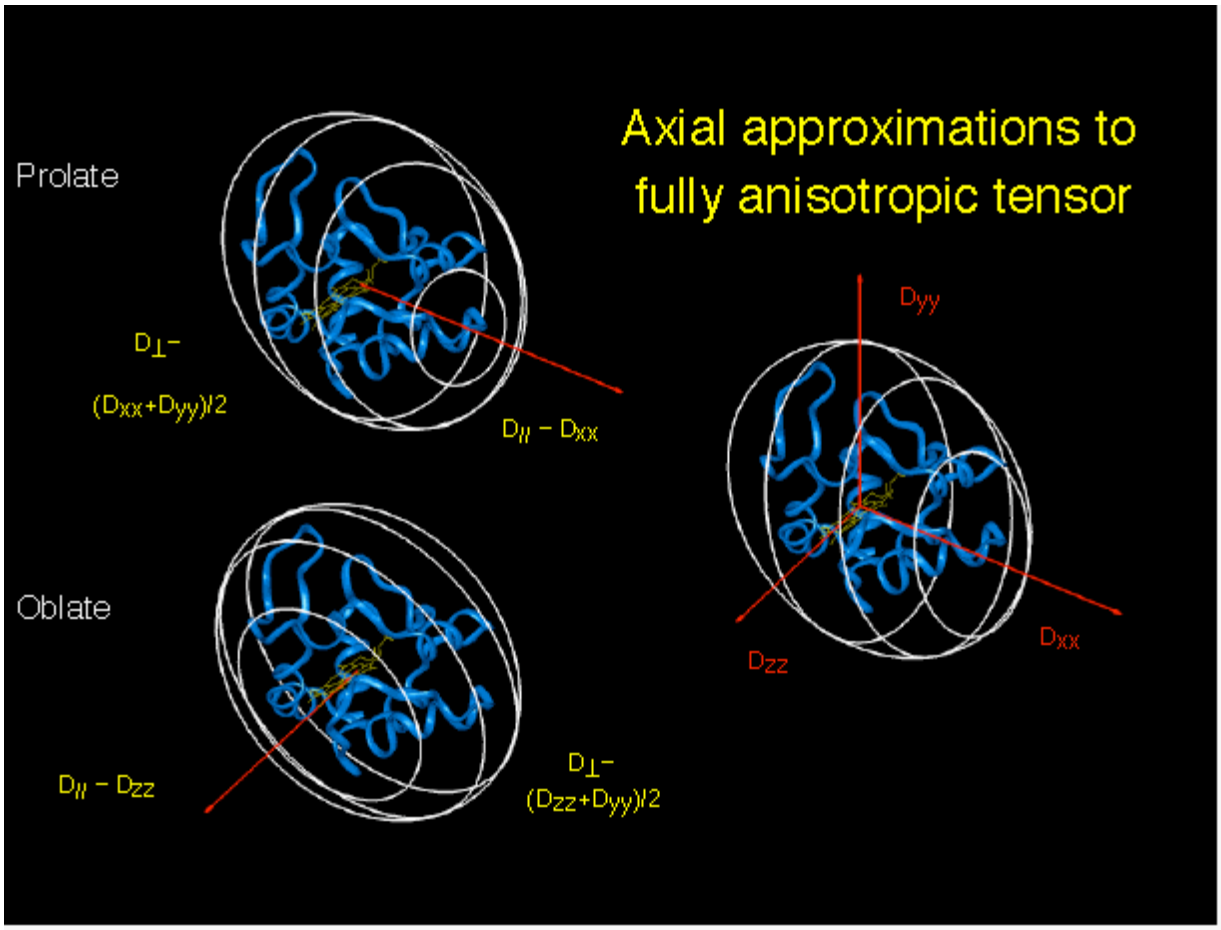


In fact, as shown below, two physically reasonable minima can exist in the fit using the axially symmetric rotational diffusion tensor, whose unique axes are related to the real axes of the asymmetric tensor by the following approximations (Blackledge et al JACS 1998): in the case of an asymmetric tensor with components (D_{xx},D_{yy},D_{zz}) the components of the axially symmetric tensor (Dper and D//) would be approximated to ((D_{xx}+D_{yy})/2, D_{zz}), and ((D_{yy}+D_{zz})/2, D_{xx}) in the three minima. The relative importance of these minima with respect to the target function is indicative of the nature of the diffusion tensor.

Below we show the target function in reduced parametric space - $\chi^2$is plotted with respect to the polar angles θ and ϕ, and the ratio D// to Dper.



The assignment of Dper or D// to the summed or the isolated term depends on the geometry of the molecule. In the case of three components which are significantly different (e.g. $D_{xx}>D_{yy}>D_{zz}$) as for the cytochrome *c*2 : we find two minima of similar significance (χ^2 = 44.3, 44.6 for the helical dataset). The orientation of the tensor in the oblate and prolate models is illustrated below (Blackledge et al 1998, Cordier et al 1998).

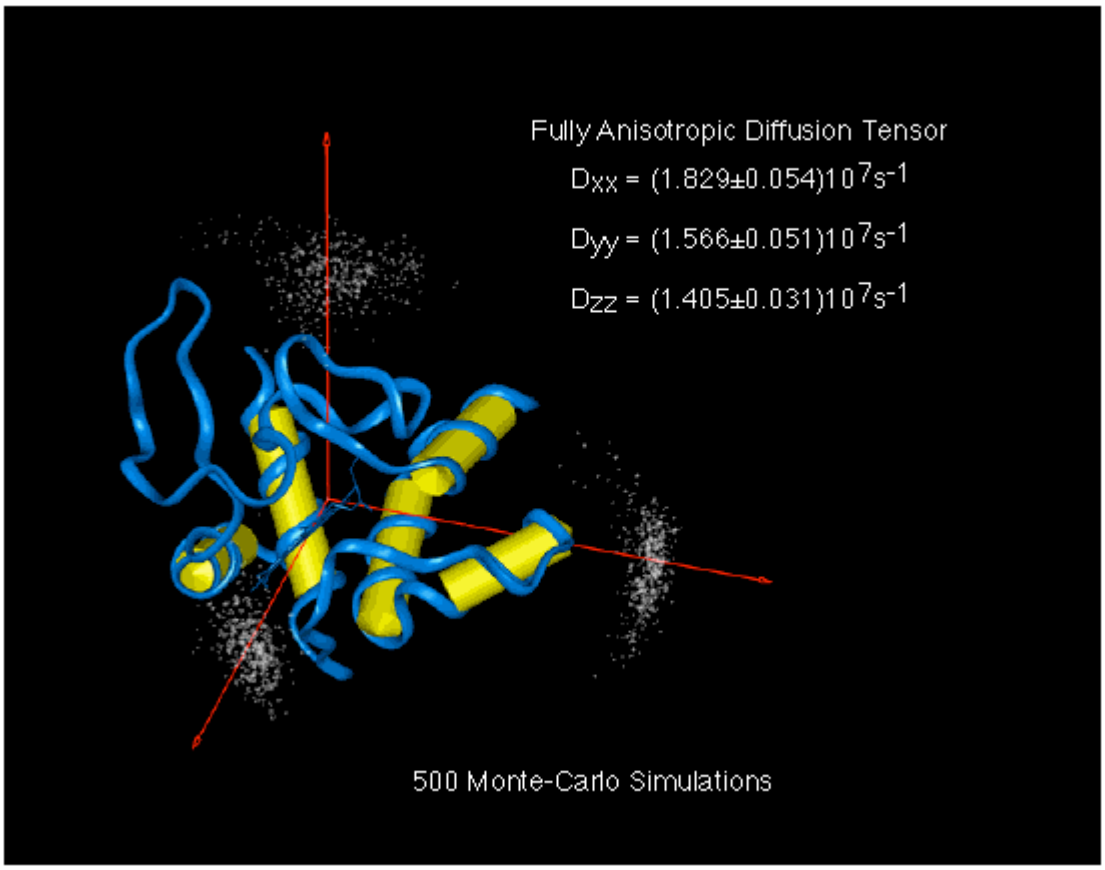


Clearly the presence of these two similarly significant minima is indicative of a rotational diffusion tensor with significant **rhombicity**. We have tested many proteins for which relaxation data and structural coordinates are available and find that this is far from a rare occurrence, but that often only one of the two minima is reported in published analyses of rotational diffusion using relaxation data. This is perhaps not surprising is we look at the χ^2 space illustrated above; depending on the values of theta and phi, equally valid minima can be steep-sided and therefore difficult to find using a grid-search type of minimisation algorithm, or broad and consequently much easier to localise. (The values of theta and phi, are of course dependent only on the reference frame, usually taken from the database coordinates, and therefore completely arbitrary.)

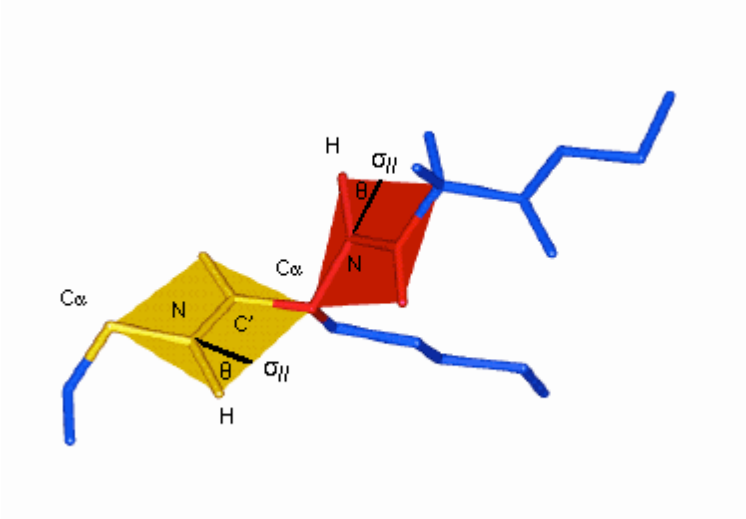
Until now, to overcome these difficulties, TENSOR systematically localized all minima (prolate, oblate and fully asymmetric) and used extensive Monte-Carlo simulations to test the relevence of the improvement available from the more complex fit using F-testing. As performed in Tensor1.0 and 1.1, and in the all of the literature we have found, this procedure is actually flawed - comparison of a model with two different minima (the axially symmetric model) with a model comprising one minimum which is approximated by the other two is not simple, and cannot be performed using the Monte-Carlo methods proposed. As noted above, the presence of two minima (even if the minima are not equally valid with respect to the target function) in the axially symmetric description is evidence of rhombicity. No further test would then be necessary. While still proposing the analysis as in Tensor1.0 and 1.1, we strongly suggest the use of the most appropriate model for an object as complex as a protein — the asymmetric rotor. Should the rotor be in reality axially symmetric, this will be reflected in the distribution of noise-simulations resulting from the Monte-Carlo simulations (see figure in Volume II - Practice).

Uncertainties in the parameterisation of the diffusion tensor are determined using Monte-Carlo simulations, based on the experimental error in the relaxation data. This allows the dispersion in both the component amplitudes and directions to be assessed, as shown for the example of

the cytochrome c_2 in the case of the fully asymmetric model.



Non-coaxiality of the csa and dipolar interactions.



For the analysis presented above, the dipolar and csa interactions were assumed to be collinear, a common approximation which is certainly not generally applicable, but which simplifies the analysis considerably.
Solid state NMR measurements and comparative studies of dipole-dipole csa cross-correlation and transverse relaxation measurements indicate values of θ, the angle in the peptide plane between the NH-csa interations, which vary between 10° and 30°; for the amide ^{15}N csa in amino acids.
We have incorporated the possibility of changing this angle from its default value of 0°;. For simplicity we assume that the same angle prevails for each residue (or that an average interaction angle is used) and that the ^{15}N csa tensor is axially symmetric and in the C_{i-1}-N_iH_i plane.
The internal motion was assumed to be identical for the two interacations, a hypothesis which is certainy not justified, even in the case of crankshaft-only motions, but one which only weights the relative significance of the dipolar and csa dependent contributions which are in any case defined by the experimentally ill-defined constants ($\sigma_{//}$-σ_{per}) and ($1/r^6_{NH}$).

Internal motion.

Measurement of heteronuclear relaxation rates have been widely used to investigate the backbone dynamics of proteins. Commonly, ^{15}N R_1 and R_2 autorelaxation rates and the heteronuclear ^1H-^{15}N NOE are measured for each available residue in the protein. Data are normally interpreted using the abstract modelfree dynamics formalism (Lipari & Szabo,1982) or by simple matrix inversion to provide direct samples of the spectral density function (Peng & Wagner,1992,1995) of NH vectors along the peptide chain. In the modelfree approach mobility is characterized using an order parameter S^2, which may be interpreted as the amplitude of the motion and a correlation time τ_i- the characteristic time constant of this motion.

The physical nature of the mobility is not constrained, but the internal and global motion are assumed independent and the overall and internal autocorrelation functions are assumed to have an exponential nature. The interpretation has been further extended to take account of two uncoupled internal motions and hence three independent terms in the time correlation function (Clore et al., 1992).

This approach has the advantage of being quantitative, intuitive and relatively easy to test for statistical significance (Mandel et al., 1995). Although non-specific and valid only in restricted timescales with respect to overall motion, this simple analysis has gained in popularity with the routine investigation of ^{15}N labelled proteins to become a standard measure in the solution study of proteins using heteronuclear nmr spectroscopy.

Relaxation Data Analysis for Internal Mobility.

Lipari-Szabo Approach.

Internal and global motion are assumed to be **independent** and the autocorrelation function is described by the two **exponential** processes - internal motion is defined by two parameters describing the amplitude (**S^2** - called the square of the order parameter)

and a characteristic correlation time of the motion τ_i .

$$C_{total}(\tau) = C_i(\tau).C_g(\tau)$$

$$C_i(\tau = 0) = 1 \;,\; C_i(\tau \to \infty) = S^2 \text{ and } \int_0^\infty C_i(\tau) = \tau_i$$

$$C_i(\tau) = S^2 + (1 - S^2)e^{-\tau/\tau_i}$$

The plateau value of the correlation functions S^2 ($0 \le S^2 \le 1$) describes the spatial restriction of the X-H vector, and τ_i the internal correlation time of this vector in the molecular frame.

In the case of isotropic overall motion $C_g(\tau)$ is again given by ;

$$C_g(\tau) = \frac{1}{5}e^{-\tau/\tau_c}$$

Limiting cases -

S^2 approaches 0; relaxation described only by internal motion.

S^2 approaches 1; relaxation described only by global motion.

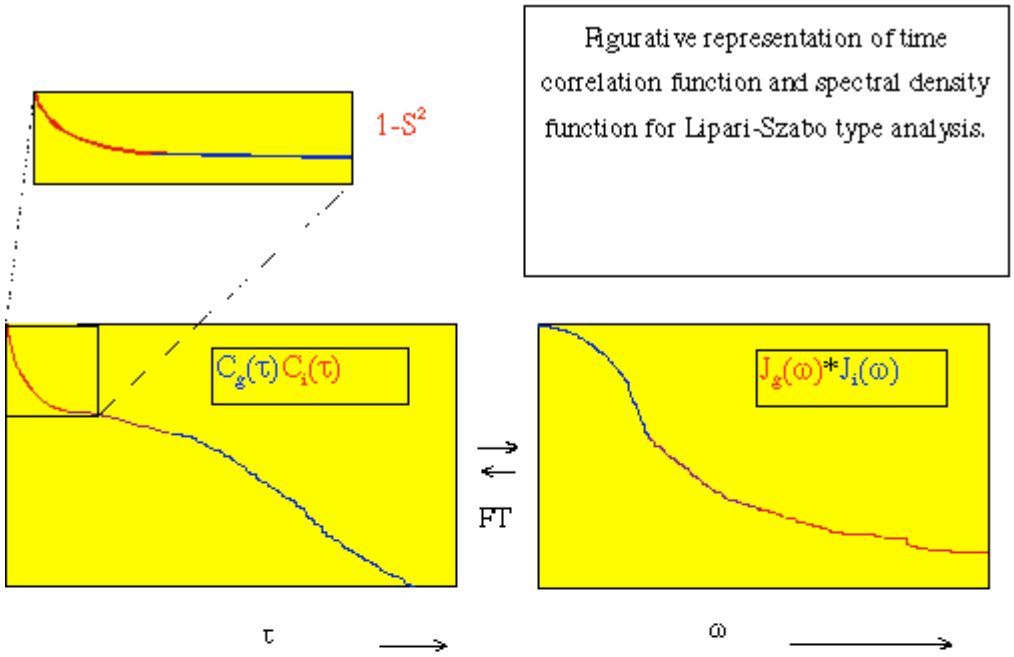
Similarly the **spectral density function** can be described using this formalism -

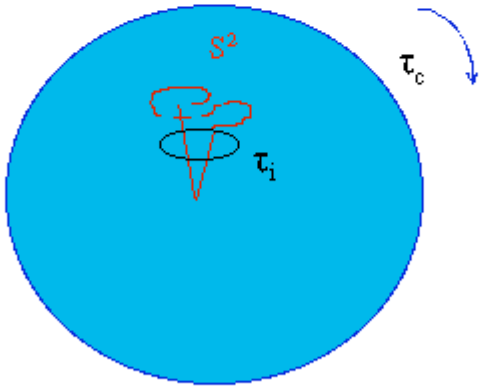
$$J(\omega) = \frac{2}{5}\left[S^2\frac{\tau_c}{1+\omega^2\tau_c^2} + (1-S^2)\frac{\tau_e}{1+\omega^2\tau_e^2}\right]$$

where $\tau_e^{-1} = \tau_c^{-1} + \tau_i^{-1}$, τe is the effective internal correlation time and τc is the isotropic global correlation time. In the limit $\tau e << \tau c$ and $S^2 \to 1$

$$J(\omega) = \frac{2}{5}\frac{S^2\tau_c}{1+\omega^2\tau_c^2}$$

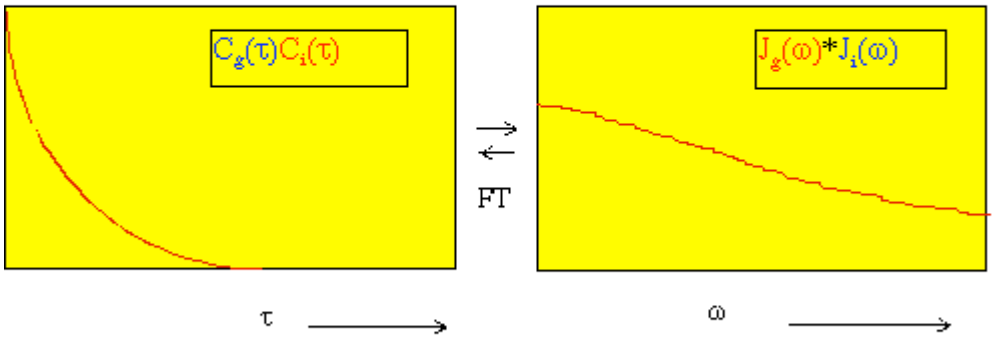
Figurative representations of time correlation and spectral density functions.

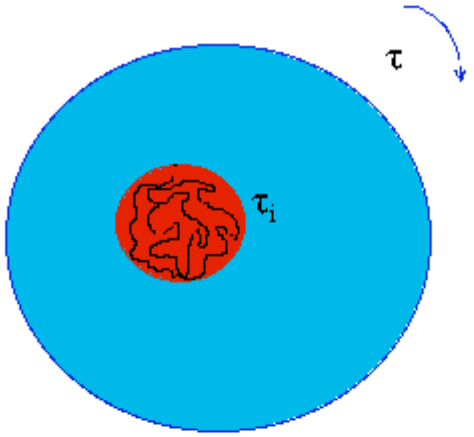




If the internal motion is restrained (not isotropic) then relaxation will be affected by both local and global motion.

Figurative representation of time correlation function and spectral density function for isotropic local mobility (S^2=>0).





If the internal motion is completely unrestrained (isotropic) then the relaxation is only dependent on local motion.
No information is available concerning global reorientation because the autocorrelation function is dominated by more rapid reorientation - irreversible loss of **order**.

The spectral density function applicable in the relaxation rate equations can thus be defined (in its simplest form) for **all** J(ω) terms by **two** parameters describing the internal motion (S^2, τ_i) and by one term (τ_c) describing the overall motion.

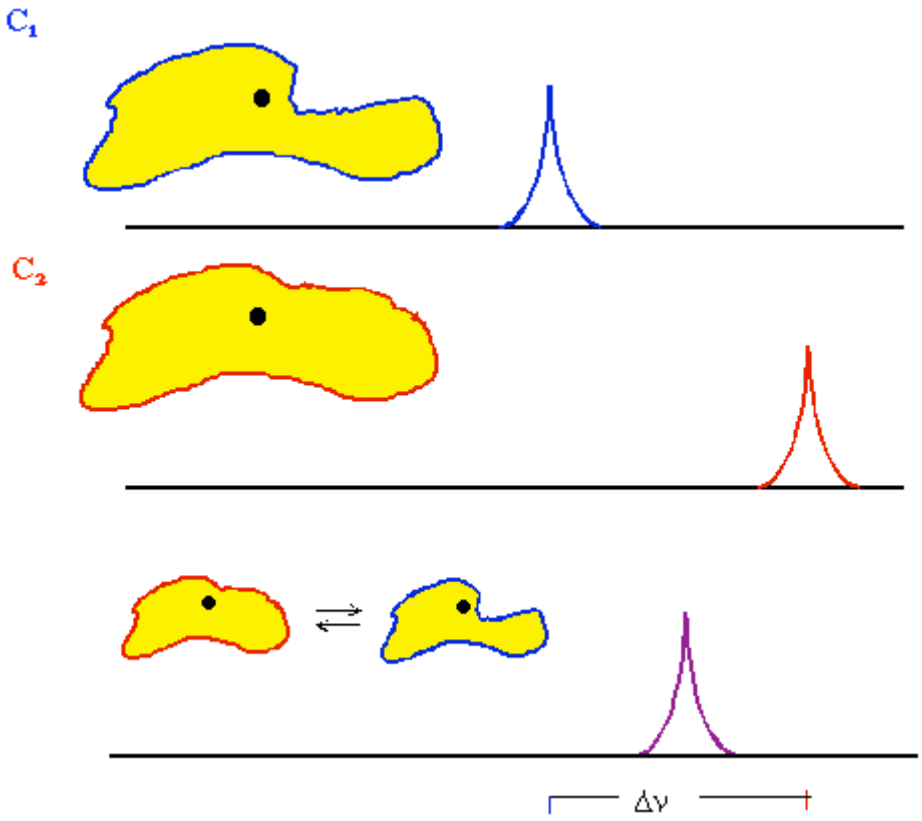
This spectral density function is then a non-linear function with respect to (S^2, τ_i)
It is necessary to fit the relaxation data by non-linear least squares optimisation of a target function -

$$\chi^2 = \sum[R_x(S^2, \tau_i, \tau_c) - R_x(exp)]/\sigma_x^2$$

where R_x represents the measured relaxation rates R_1,R_2 and nOe.

In the case of isoptropic overall tumbling, the parameter $\tau_c$is common to all points in the molecule - this formalism allows for three measured variables to characterise the internal mobility using two parameters.

Exchange Term (3, 4). For specific sites in the molecule it may be necessary to supplement these two parameters {(S^2, τ_i} with a chemical shift exchange contribution Rex, to the measured transverse relaxation rate R2, due to rapid (μs-ms) interconversion of sites experiencing varying chemical envirnments.



Conformational Exchange - (τ_{exch} - μs)

$$R_2 (eff) = R_2(dip) + R_2(csa) + R_2(exch)$$

Transverse relaxation can have a component due to exchange between two chemical shift sites

$$R_2(exch) = \frac{4\pi\Delta\nu^2 P_1 P_2 \tau'_{exch}}{1 + \omega_1^2 \tau'^2_{exch}}$$

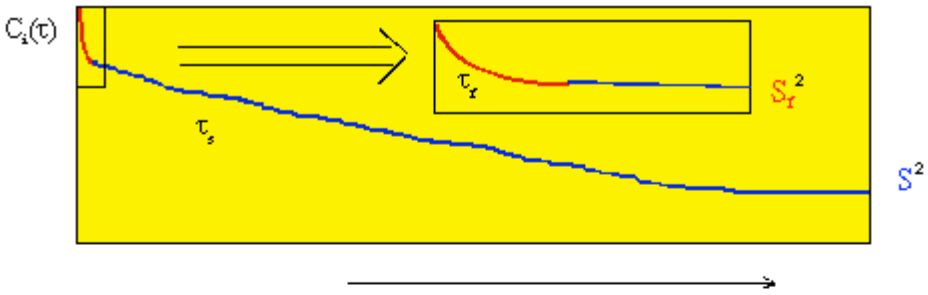
Extended Lipari-Szabo Model (5).

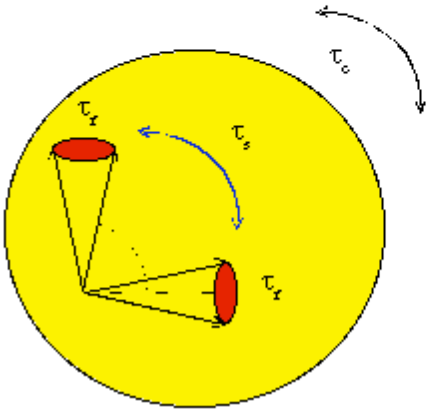
It is sometimes necessary introduce additional movement with an intermediate correlation time to explain the relaxation data

$$C_i(\tau) = S^2 + (1 - S_f^2)e^{-\tau/\tau_f} + (S_f^2 - S^2)e^{-\tau/\tau_s}$$

the movement is parametrised by τ_f and τ_s and two order parameters S_f^2 and S_s^2 for the fast and slow motions respectively. $\tau_f << \tau_s << \tau_c$. $S^2 = S_s^2 S_f^2$.

The correlation function drops rapidly to a plateau (S_f^2) then more slowly to a second plateau (S^2).





Movement of X -H vector, considering two internal motions ($\tau_f << \tau_S << \tau_c$).

The slow motion can be for example a two-site jump, and the more rapid, diffusion in a cone superposed on this movement.

The full spectral density function :

$$J(\omega) = \frac{2}{5}\left[S^2 \frac{\tau_c}{1 + \omega^2\tau_c^2} + (1 - S_s^2)\frac{\tau'_s}{1 + \omega^2\tau'^2_s} + (S_s^2 - S^2)\frac{\tau'_f}{1 + \omega^2\tau'^2_f}\right]$$

where $\tau_f'^{-1} = \tau_c^{-1} + \tau_f^{-1}$ et $\tau_s'^{-1} = \tau_c^{-1} + \tau_s^{-1}$ are the effective correlation times of the two motions ($\tau_x << \tau_s << \tau_c$).

With $\tau_x \rightarrow 0$, the equation reduces to :

$$J(\omega) = \frac{2}{5}\left[S^2 \frac{\tau_c}{1+\omega^2\tau_c^2} + (S_s^2 - S^2)\frac{\tau_s'}{1+\omega^2\tau_s'^2}\right]$$

The internal motion is parametrised by S_s^2, S_f^2 and ts.

Now we see why we have to be very careful with this type of analysis — there are N(measurables) and N(parameters)!!!!!!!!

Statistical Analysis of Heteronuclear Relaxation Data

Measurements - (R_{1i}, R_{2i}, nOe_i)

Dynamic parameters - τ_c(S^2_2, S^2_1, τ_i, R_{ex})

It is clear from the above, that the Lipari-Szabo approach can be severely underdetermined if only the measurements (R_{1i}, R_{2i}, nOe_i) are available for each residue i.

Data analysis must be based on strict statistical analysis to avoid overinterpretation.

This is achieved by applying the most simple model and iteratively adding dynamic parameters until the fit is statistically significant.

Estimation of τ_c.

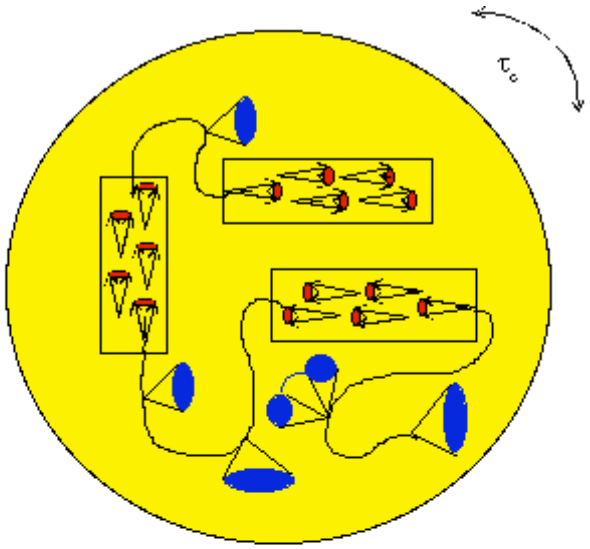
It has been proposed that the (R2i /R1i,) ratio of the **most rigid** vectors in the molecule can provide an initial estimate of τ_c.

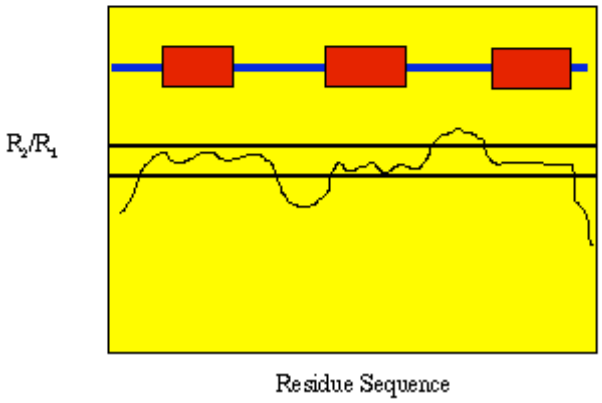
This is valid because -

$$J(\omega) \approx \frac{2}{5}\frac{S^2\tau_c}{1+\omega^2\tau_c^2} \text{ as } \tau i \text{ Æ } 0$$

$$\frac{R_{2i}}{R_{1i}} \approx \frac{\frac{d}{8}\times\{4J(0)+J(\omega_H-\omega_N)+3J(\omega_N)+6J(\omega_H)+6J(\omega_H+\omega_N)\}+\frac{c}{6}\times\{4J(0)+3J(\omega_N)\}}{\frac{d}{4}\times\{J(\omega_H-\omega_N)+3J(\omega_N)+6J(\omega_H+\omega_N)\}+c\times\{J(\omega_N)\}}$$

and the ratio (R_{2i} /R_{1i},) becomes independent of τ_i and S^2. It is also necessary that these residues do not experience chemical exchange, which could increase R_{2eff}.





R_{2i}/R_{1i}, is then, to a first approximation, only a function of overall tumbling τ_c.

Determination of isotropic overall correlation time τ_c.

Analysis of internal mobility using TENSOR2.

Isotropic Global Rotational Diffusion and Internal Mobility.

The spectral density function we will use in TENSOR2 is given by;

$$J(\omega) = S_2^2\left\{S_1^2\frac{\tau_c}{1+(\omega\tau_c)^2} + (1-S_1^2)\frac{\tau'}{1+(\omega\tau')^2}\right\} \quad \text{with} \quad \tau' = \frac{\tau_c\tau_i}{\tau_c+\tau_i}$$

Note that this is slightly different from that used in the explanation given above — there is a factor 2/5 missing here. This definition is correct for the relaxation rate equations quoted above.

Using the Lipari-Szabo model-free approach J(ω) is derived assuming $S_2^2 = 1$. The generalized order parameter $S_1^2 = S^2$ describes the amplitude of the fast internal motion and τ' the effective correlation time for fast internal motions. A second slower internal motion may be necessary - this movement is characterized by $S_1^2 = S_s^2$ and τ_i; the order parameter and internal correlation time for the slow internal motion. The correlation time of the fast motion is assumed to be negligible with regard to the measured relaxation parameters which are only affected by the order parameter $S_2^2 = S_f^2$ ($S^2 = S_s^2 S_f^2$) describing the rapid librational motion.

Analysis initiated by estimating the overall correlation of the molecule, either using the isotropic approximation, characterized by the time τ_c, or by determining D, the rotational diffusion tensor, both described above.

Determination of Internal Motional parameters.

The following five models are iteratively tested starting with the simplest model and invoking more complex models until the proposed model could give rise to the measured relaxation rates within 95% confidence limits: (1) S^2; (2) S^2, τ_i ; (3) S^2, R_{ex}; (4) S^2, τ_i, R_{ex}; and (5) S_f^2, S_s^2, τ_i. In model (1), motions on the fast timescale are too fast (< 20 ps) to be characterized and affect R_1 and R_2 in a similar way. In model (2), internal motion is relaxation active. In models (3) and (4) R_{ex} contributes to R_2. Model (5) refers to the extended model described

above.

This rigorous analysis is designed to avoid over-interpretation of relaxation data in terms of fictive internal motions, and is very strongly inspired, if not directly copied from the analysis puiblished by Professor Palmer's group (Mandel et al 1995) and available with the program MODELFREE.

Available Models for internal dynamics - Summary ;

Following the expression for J(ω) given above, five models of internal mobility are possible, assuming **D** (rotational diffusion tensor) is known.

1 - we consider that τ_i is very fast (τ_i<20ps) -

$$J(\omega) = \frac{2}{5}\frac{S^2\tau_c}{1+\omega^2\tau_c^2}$$

2 - τ_i is relaxation active (classical Lipari Szabo)

$$J(\omega) = \frac{2}{5}\left[S^2\frac{\tau_c}{1+\omega^2\tau_c^2} + (1-S^2)\frac{\tau_e}{1+\omega^2\tau_e^2}\right]$$

3 - J(ω) as for M1 with a chemical exchange contribution.

4 - J(ω) as for M2 with a chemical exchange contribution.

5 - Extended Lipari Szabo model, including a very fast and a slower internal motion ($S_1^2 = S_s^2 \neq 1$ and τ_s and, $\tau_f \rightarrow 0$).

$$J(\omega) = \frac{2}{5}S_2^2\left[S_1^2\frac{\tau_c}{1+\omega^2\tau_c^2} + (1-S_1^2)\frac{\tau_e}{1+\omega^2\tau_e^2}\right]$$

Dynamic Parameters	
Model 1	S^2
Model 2	S^2, τ_i
Model 3	S^2, R_{exch}

Model 4	S^2, τ_i, R_{ex}
Model 5	S_z^2, S_{xy}^2, $\tau_i = \tau_s$

Sets of relaxation data are fitted to the different dynamic models, using the optimized global correlation function by minimizing the function:

$$\chi^2 = \sum_i \left\{ \frac{R_{i,n}^{meas} - R_{i,n}^{calc}}{\sigma_{i,n}^{meas}} \right\}^2$$

where i represents the relaxation parameters used in the calculation. $R_{i,n}^{meas}$, $R_{i,n}^{calc}$ and $\sigma_{i,n}^{meas}$ are the experimental and calculated relaxation rates/nOe and the estimated experimental uncertainty of $R_{i,n}^{meas}$ respectively.

The five models are tested iteratively in order of increasing complexity until the model reproduces the data adequately (95% confidence limits). Model selection is based on Monte-Carlo simulations to characterize the random variation in the fit, to provide probability statistics and estimate uncertainty. An F characteristic is used to judge the statistical significance of introducing an additional parameter to model (1). F is defined as

$$F = \frac{(N-n)(\chi_m^2 - \chi_n^2)}{(n-m)\chi_n^2}$$

for the comparison of models fitting N variables with m and n parameters. In the case where the reduction of $\chi^2$is less than the α=0.20 critical value for random statistical improvement we reject the more complex model and propose the model (1) parameterization.

Rotational Diffusion Anisotropy and Internal Mobility.

The orientation and the components of the diffusion tensor can be introduced into the first term of the general spectral density function:

$$J(\omega) \approx S^2 \sum_j \frac{A_j \tau_j}{1+(\omega\tau_j)^2} + (1-S^2)\frac{\tau'}{1+(\omega\tau')^2} \quad \text{with } \tau'^{-1} = 6D + \tau_i^{-1}$$

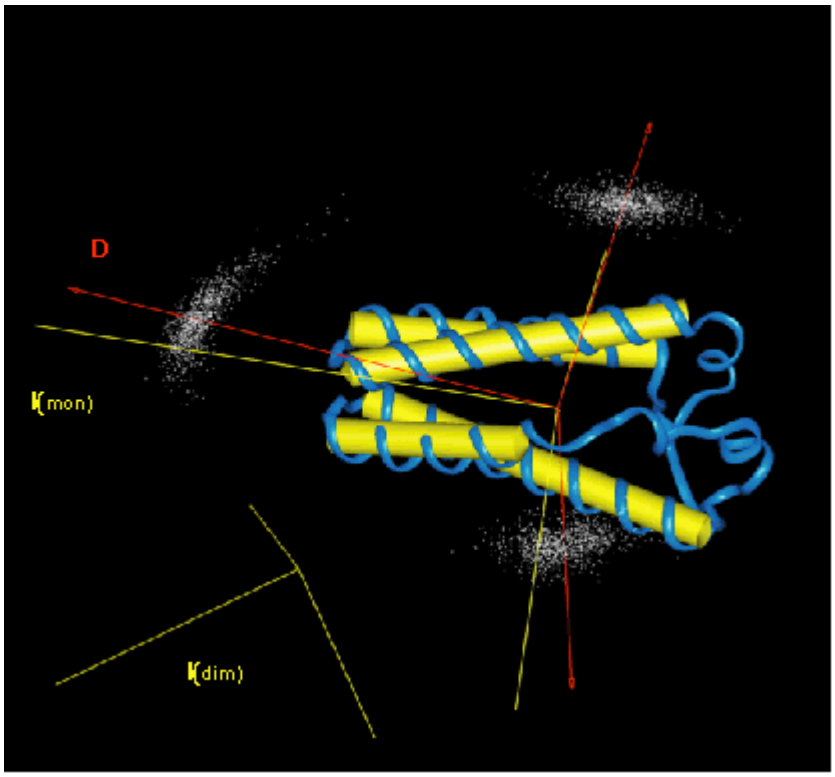
This definition can again be used to characterize the local mobility in terms of the Lipari-Szabo formalism. Fast internal motion ($\tau_i << (6D)^{-1}$) is assumed to be independent of overall rotational tumbling and is thus analogous to the isotropic Lipari-Szabo modelfree approach. Statistical testing of the significance of the derived models was determined using identical criteria for model selection as in the isotropic case.

Example - Cytochrome c' from *Rhodobacter Capsulatus*.

As an example of the possible advantages if using the anisotropicrotational diffusion tensor to describe overall movement of the molecule, rather than the simpler isotropic tensor, we have

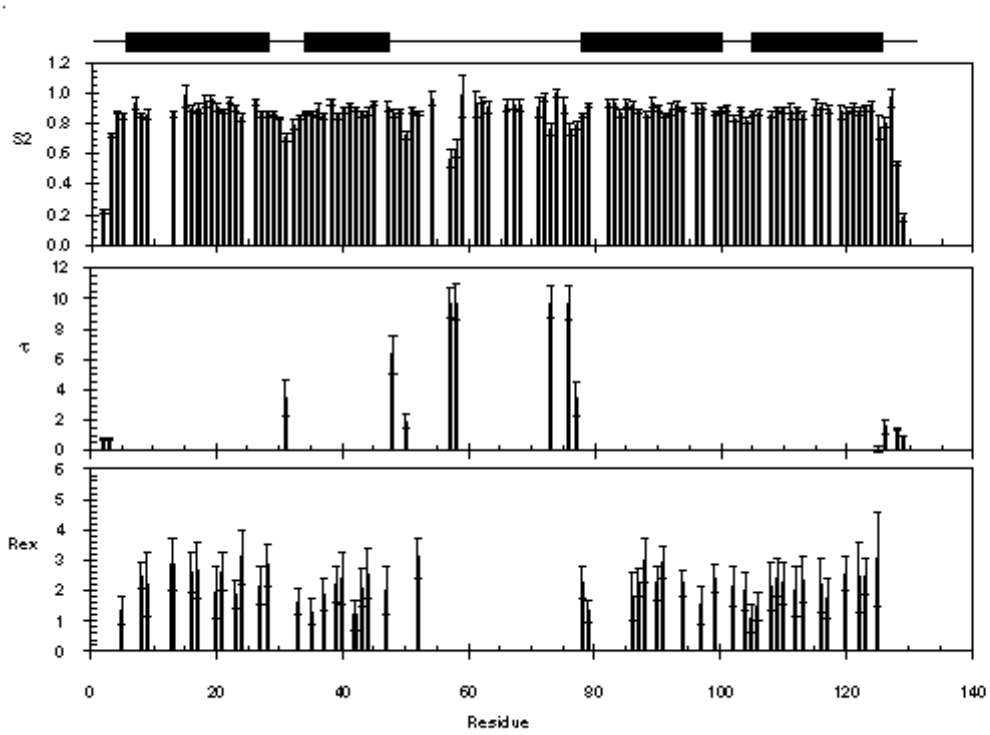
chosen the Cytochrome c' from *Rhodobacter Capsulatus,* which we have recently published in the *Journal of the American Chamical Society* (2000).

The determination of the rotational diffusion tensor already told us something important about the molecule - It has been suggested that this protein exists as a dimer in solution. We compared the diffusion tensor to expected inertia tensors of the proposed (crystallographic) dimer orientation and the monomer. As shown in the figure below this showed quite clearly that the molecule is more likely to be a monomer, given the strong similarity between the inertia tensor orientation of the monomer and the diffusion tensor, and the dissimilarity to the dimeric inertia tensor



Possibly more importantly, the Lipari-Szabo analysis of internal mobility in the protein, calculated using TENSOR2, gave strikingly different results if the molecule was assumed to tumble isotropically, or if we took the tensor shown above to be the correct description of overall diffusion.

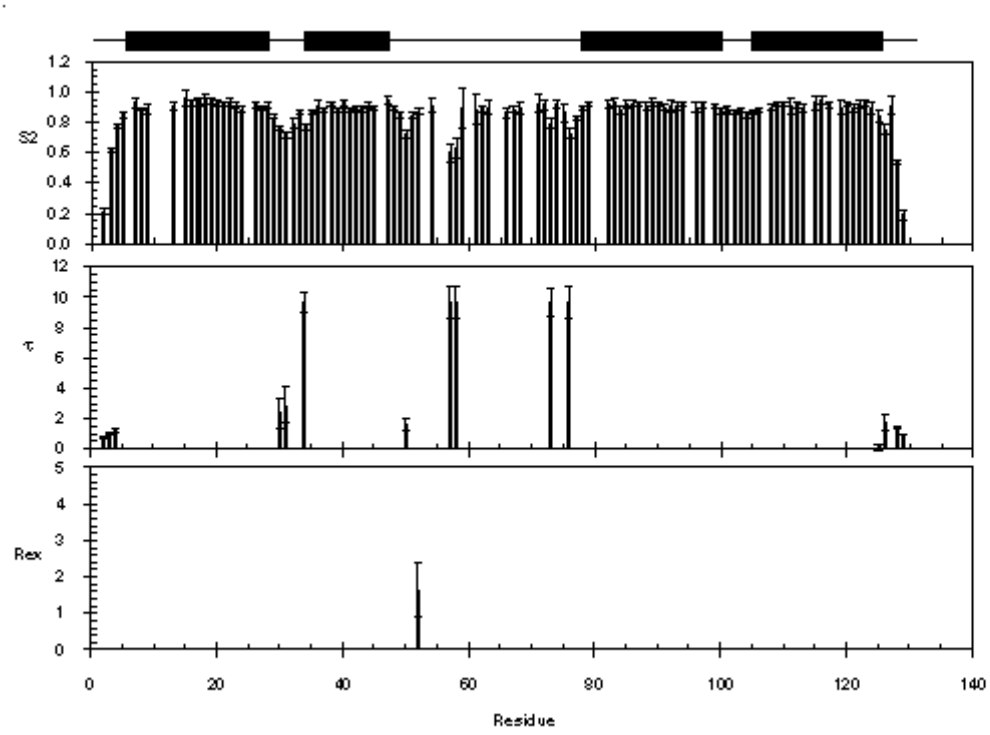
Internal motion calculated assuming Isotropic overall tumbling ;



Note the presence of chemical exchange contributions in the 4 alpha-helices, and more complicated motion in the intermediate loop region. If we look closely we can actually see that the exchange contributions are periodic within the helices. Indeed the order parameters also exhibit a periodicity. All of these manifestations of apparently complicated internal motion actually turn out to be entirely artefactual, and due simply to the inappropriate model we have used for the overal rotational tumbling.

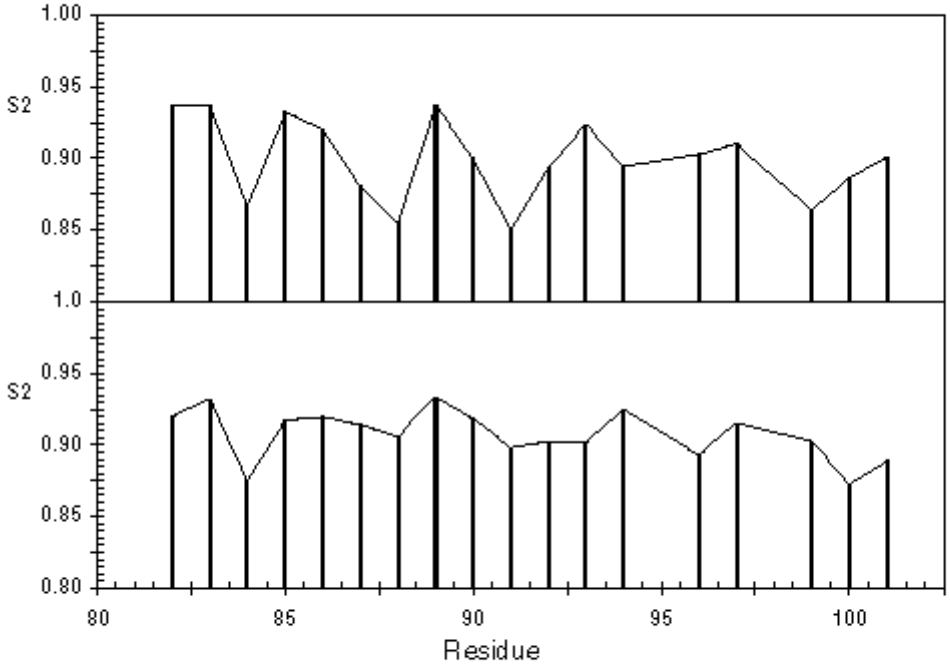
Using the anisotropic rotational diffusion tensor determined from residues present in secondary structural motifs, we find (not surprisingly) that the description of internal mobility is greatly simplified.

Internal motion calculated using the anisotropic diffusion tensor determined from TENSOR2;



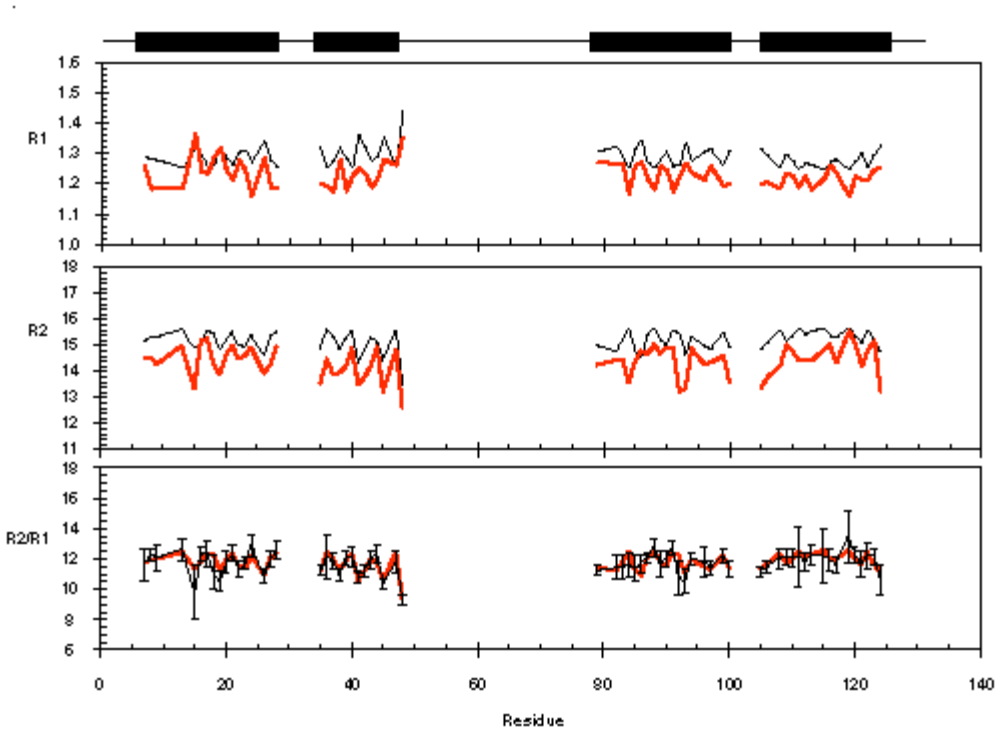
We can see that the exchange contributions have disappeared (so we are no longer evoking periodic motion along each helix on a micro-milli second timescale), and that the order parameters in the helices are actually flatter as well. This is shown below for helix III:

Top - Order parameter determined for helix III assuming isotropic tumbling.
Bottom - Order parameters for helix III using the anisotropic diffusion tensor to describe overall tumbling.



The reason for this periodicity (both in the chemical exchange and order parameter) is the varying orientation of the relaxation mechanisms (of the NH vectors to a first approximation) relative to the anisotropic diffusion tensor. An NH bond makes an angle of 13° with the axis of the helix, so that a significant range is sampled by the vectors as we move along a helix. This affects the relaxation rates (see below for the measured values in the helices of both transverse and longitudinal relaxation rates). If we ignore the dependence of the relaxation rates on the orientation of the vectors relative to the diffusion tensor, we will be obliged to evoke complicated internal mobility to compensate for this error. This is exactly what we do if we assume that the molecule tumbles isotropically (all relaxation mechanisms due to overall tumbling of the molecule are equivalent, regardless of orientation).

Experimental R1 and R2 rates and R2/R1(measured at 600MHz) shown in red, compared to optimal values determined from the fit of the anisotropic diffusion tensor D, shown in black. Note that the inverse dependence of R1 and R2 on orientation with respect to D, makes R2/R1 particularly sensitive. This is because the values of $\{1/\tau_{1\text{-}5}\}$ lie between $\omega=0$ and ω_N.



ps - I realise that all data is not this precise, but an accurate description of rotational tumbling will help under any circumstances!

Statistical Analysis.

The criteria used for acceptance of proposed models are 95% confidence tests comparing the experimental $\chi 2$ to $\chi 2$ distributions based on fitting of simulated datasets from Monte-Carlo sampling of gaussian distributions. These are defined by the experimental uncertainty and centered on the relaxation parameters back-calculated from the best-fit. We have also included the use of the F characteristic to judge the statistical significance of invoking an additional parameter to the axially symmetric model, compared to the random statistical improvement characteristic of the increased available degrees of freedom. F is defined as

$$F = \frac{(N-n)(\chi^2_m - \chi^2_n)}{(n-m)\chi^2_n}$$

for the comparison of models fitting N variables with m and n parameters. Both χ^2 and F distributions calculated by TENSOR2 can be inspected graphically.

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