Measurement of amyloid fibril length distributions by inclusion of rotational motion in solution-state NMR diffusion measurements

Andrew J Baldwin, Spencer J Anthony-Cahill, Tuomas PJ Knowles, Guy Lippens, John Christodoulou, Paul D Barker, Christopher M Dobson*

Supra-molecular assemblies and other nanoscale structures fall within a range of characteristic length scales where, in contrast to the situation for smaller molecular species, rotational motion provides an important contribution to particle displacement in addition to translational diffusion \[1\]. Here we demonstrate that this effect not only has profound implications for the interpretation of NMR diffusion experiments, but also can also be used to provide the basis for a new approach for measuring in situ length distributions of high-aspect ratio molecular assemblies, such as amyloid fibrils. The ability to make such measurements is of very considerable importance as accurate length determination of biological filaments in solution remains an extremely challenging task, and the commonly used microscopy-based approaches require transfer of the structures from solution to a surface prior to analysis. We have studied fibrils of an SH3 domain fused to apo-cytochrome b562 (apo-SH3-Cyt) \[2\] and demonstrate that the lengths obtained using our NMR method are comparable to, but slightly larger than, those measured using atomic force microscopy (AFM) and transmission electron microscopy (TEM) following deposition on a surface. We discuss the implications of this finding for the definition of the sizes of nanoscale biological components in solution.

The flexible regions of a number of large biomolecular systems, including multi-domain enzymes,\[3,4\] molecular chaperones, \[5\] intact ribosomes,\[6,7\] and non-core residues of amyloid fibrils\[2,8,9\] have been characterized by solution-state NMR spectroscopy. Although the size of such species can greatly exceed the usual limits for solution-state NMR methods, the ‘motional narrowing’ resulting from local mobility can be sufficient to average almost completely the internal dipolar interactions that normally dominate the transverse relaxation rates.\[2,8\] NMR resonances from such states may then have linewidths comparable to those of free peptides in solution, making possible the application of a wide range of NMR techniques.

One type of experiment of particular value for the study of large complexes involves the use of pulsed field gradients (PFGs) to measure translational diffusion coefficients and hence estimate their molecular dimensions.\[10\] In studies of amyloid fibrils, however, we observed anomalies in such measurements. We prepared and purified fibrils of apo-(SH3)-Cyt \[2\] as described in S1. TEM analysis revealed structures typical of amyloid assemblies with widths of ca. 10nm, and a range of lengths in the vicinity of 2μm (Fig 1A). These fibrillar species \[2\] were found to yield well resolved solution-state NMR spectra a situation that can be attributed to be due to the fast dynamics of the substantially unfolded cytochrome component in the fibrils (Fig 1B). Pulsed field gradient stimulated echo (PFGSE) NMR data were then recorded with a conventional diffusion delay (Δ=100ms) and analysed using the Stejskal-Tanner (ST) equation, \[11\]

\[
D_{\text{eff}} = \frac{1}{\alpha^2 \beta} \ln \frac{S_i}{S_0} \tag{1}
\]

where \(D_{\text{eff}}=D_T\). This equation relates the signal intensity in the presence, \(S_i\), and absence, \(S_0\), of the applied gradients of field strength \(G\), measured as a fraction of the maximum gradient that is applied, \(G_{\text{max}}\) such that \(G=G/G_{\text{max}}\), to the translational diffusion coefficient \(D_T\) of the sample under study, where \(\alpha=\gamma \delta G_{\text{max}}, \beta=\Delta / 3, \gamma\) is the gyromagnetic ratio of the nucleus under study, \(\delta\) is the gradient duration and \(\Delta\) is the diffusion delay. The values of \(D_T\) calculated in this manner were found to be sample dependent (Fig 1C) and the values of \(R_q\) (Fig 1D, 2-12nm) determined from \(D_T\) using the Einstein-Stokes equation (S2), were approximately three orders of magnitude less than the fibril lengths observed by TEM.

When NMR spectra were acquired with an exceptionally long diffusion delay (Δ=1s), the resonances of monomeric apo-(SH3)-Cyt were clearly observed at 10% \(G_{\text{max}}\) but were entirely suppressed by 50% \(G_{\text{max}}\) (Fig 1B, top). Remarkably, only modest attenuation of the signal intensity was observed between the spectra of fibrillar apo-(SH3)-Cyt at 10% and 50% \(G_{\text{max}}\) (Fig 1B, bottom). Fitting these data as above yields an \(R_q\) value of 165 nm, a result inconsistent both with the fibril lengths observed by TEM and the \(R_q\) estimate obtained with \(\Delta=100ms\).

PFGSE NMR experiments measure directly the net physical displacement of nuclear spins.\[10\] For large systems where at least one length dimension exceeds 500 nm, displacement due to rotational diffusion is similar to, and indeed can exceed the contribution from translational diffusion (S3). Thus, the influence of rotational motion must be considered explicitly when analyzing NMR diffusion data for large systems. In order to interpret the data for the amyloid fibrils studied in the present work, we first consider

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* A.J. Baldwin, S.J. Anthony-Cahill, J. Christodoulou, P.D. Barker, C.M. Dobson
Department of Chemistry
University of Cambridge
Lensfield Road
Cambridge, CB2 1EW (UK)
Fax: (+44) 1223 336362
E-mail: cmd44@cam.ac.uk

T.P.J. Knowles
Centre for Nanoscience and Cavendish Laboratory
University of Cambridge
Madingley Road,
Cambridge, CB3 0FF (UK)

G. Lippens
UMR8576
CNRS
59655, Villeneuve d’Ascq (France)

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Supplementary Information

S1 - Methodology

Materials

Apo – (SH3)2Cytb562 was expressed and purified from E.coli as described elsewhere1. Fibrils were formed at low pH and purified by ultracentrifugation at 290k g for 1 hour in a Beckman-Coulter TLA-120 rotor, such that 10S and larger species were sedimented. The pellet fraction was resuspended in 3 mM HCl and the cycle repeated to ensure smaller monomeric and oligomeric protein has been removed.

NMR

NMR data were acquired on Bruker Avance500 and 700 instruments equipped with cryoprobes. Sample concentrations varied between 20 and 100 µM protein, calculated in terms of total number of monomeric units. The sample analysed in particular detail (figure 2 of the main manuscript) was at a concentration of 60µM. The spectra of individual samples were unchanged when recorded at different times over periods of weeks. Re-purification of a previously purified fibril samples by ultracentrifugation, showed only trace material (<1%) in the second supernatant, demonstrating that the monomer/fibril exchange timescale is much slower than the timescale of the experiment.

The pulsed field gradient stimulated echo (PFGSE) sequence with 3-9-19 water suppression (Bruker pulse sequence stebpgp1s19) was used for the diffusion experiments with p30=2.7ms (hence δ=2×p30=5.4ms). The diffusion delay, Δ was varied as described in the manuscript. Corrections for sample viscosity were made by simultaneously measuring the self-diffusion of water. For a ‘conventional’ diffusion experiment, d20 was set to 100ms. Processing of FIDs was performed using NMRPipe2, performed in batch using scripts written using c shell. Numerical analysis of the raw NMR data and subsequent fitting to the diffusion model were performed using in-house software programme in C++, making use of the GNU Scientific Library (GSL)3, with outputs visualised using Gnuplot, via scripts generated by the program. Source code is available on request.

The intensity profiles shown were obtained by integrating signal intensities of high S/N peaks in the spectrum. By comparing the variation of measured decay constants between individual recorded frequencies one can estimate experimental uncertainties. Estimating the uncertainty in this way gives comparable results to the uncertainties obtained from repeating experiments to calculate the standard error.

TEM

Bright field TEM images were taken on a Philips CM100 transmission microscope operating at 80kV. Contrast between sample and substrate was achieved with uranyl acetate negative staining. Typically 25 pmoles of fibril were added to UV irradiated formvar and carbon coated 400 mesh nickel grids. The fibrils were sufficiently spaced so that length distributions could be determined using ImageJ4.

AFM

AFM data were acquired using a Molecular Imaging Pico Plus microscope operating in AC-AM mode (tapping mode) in air. Ultrasharp Micromasch NCS36 silicon cantilevers were used at resonance frequency of 150 kHz. Typically 25pm of fibril in 20 µl were deposited onto a freshly cleaved mica surface. The samples were then left to dry in air for 60 min whilst shielded from dust particles. Images were analysed with ImageJ4 to determine length distributions.
S2 - The Einstein-Stokes equation

Rotational $D_R$ and translational $D_T$ diffusion coefficients are given by $D = \frac{kT}{f}$, where $f$ is an appropriate friction factor, $k$ is the Boltzman constant and $T$ is temperature. For small hard spheres, the translational friction factor is given by Stokes’ law as $6\pi \eta R_H$, where $\eta$ is the viscosity of the solution and $R_H$ is the hydrodynamic radius of the sphere. The Einstein-Stokes equation is therefore:

$$D_T^{sphere} = \frac{kT}{6\pi \eta R_H} \quad (1)$$

Diffusion coefficients obtained from PFGSE measurements can be directly related to the geometry of species under study via the friction factors. In the data shown shown in figure 1D, the NMR diffusion coefficients were calculated for each recorded frequency over a range of peaks using the ST equation. These were converted into $R_H$ estimates using the Einstein-Stokes equation, allowing a histogram to be constructed. Analysis of the histogram allowed a mean value and experimental uncertainty estimate to be determined from the FWHM the histogram, as shown in Figure 1D. Diffusion measurements from 10 independent fibril preparations are shown in figures 1C and D in the primary manuscript.

S3 - Relationship between rotational and translational displacements

The microscopic motions of particles due to translation and rotation are related through the mean squared displacement $\langle z^2 \rangle$ for translational diffusion $D_T$, and the mean squared angular displacement $\langle \theta^2 \rangle$ for rotational diffusion $D_R$ by:

$$\langle z^2 \rangle = 2D_T \Delta$$

$$\langle \theta^2 \rangle = 4D_R \Delta$$

By considering theoretical models for friction factors (Figure S1), translational and rotational displacements due to diffusion over $\Delta$ can be calculated, as shown in figure S1. Unrestrained rotational diffusion applies in the limit $\Delta \rightarrow \infty$, which represents a regime where all rotations are equally likely. These displacements are plotted and compared in figure S2.

<table>
<thead>
<tr>
<th>Geometry</th>
<th>$f_T$</th>
<th>$f_R$</th>
</tr>
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<tbody>
<tr>
<td>Sphere</td>
<td>$6\pi \eta R$</td>
<td>$8\pi \eta R^3$</td>
</tr>
<tr>
<td>Rod</td>
<td>$3\pi \eta L \frac{1}{m(L/r)}$</td>
<td>$\pi \eta L^3 \frac{6}{3 \ln \frac{L}{r}}$</td>
</tr>
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$$\langle z_{rot \text{ restrained}} \rangle \cong \sqrt{\frac{2}{3} \left(1 - \exp(-2D_R \Delta)\right)} R^2$$

$$\langle z_{rot \text{ unrestrained}} \rangle \cong \sqrt{\frac{2}{3} R^2}$$

$$\langle z_{trans} \rangle \cong \sqrt{2D_T \Delta} \quad (2)$$

Figure S1: Left - Translational and rotational friction factors for spheres of radius $R$, and rods of length $L$ and radius $r$ where $\eta$ is the sample viscosity. Right - Equations for root mean squared displacements due to rotational and translational diffusion\(^7\). These are plotted in figure S2.

We note in this analysis that:

- A rod of length $L$ will experience a greater translational rms displacement than a sphere of radius $R = L$.
- The rms displacement due to rotational diffusion becomes comparable to that of translation diffusion for rods of $1\mu m$, and exceeds it for rods longer than $2\mu m$.
- The rms displacement due to rotational diffusion becomes comparable to that of translational diffusion for spheres of radius $1\mu m$, but never exceeds the rms displacement due to translational diffusion.
Figure S2: Plots of rms displacements due to rotational and translational diffusion for spheres and rods for $T = 300K$ and $\eta = 0.1 cP$. A - rms displacement due to rotational and translational diffusion for rods and spheres, where $\Delta = 1s$. B - Ratio of rotational to translational diffusion for rods and spheres. Solid lines are for $\Delta = 1s$ and dashed lines are for $\Delta = 50ms$

- Decreasing $\Delta$ reduces the length where the rms displacement for translation becomes comparable to the rms displacement for rotation.

For rods, the limit $\lim_{L \to \infty} \frac{<z_{rot}>}{<z_{trans}>} = \sqrt{6}$ is reached where $L = 1\mu m$ for $\Delta = 100ms$ and by $L = 5\mu m$ for $\Delta = 1s$. For spheres, $\lim_{L \to \infty} \frac{<z_{rot}>}{<z_{trans}>} = \frac{1}{2}$. This limit is reached for spheres of radius 200nm where $\Delta = 100ms$, and for spheres of radius $1\mu m$ where $\Delta = 1s$. In this limit, the formula for restricted rotational diffusion should be applied. Thus the relative contribution of rotational motion to the total rms displacement will be greater in general for a rod than a sphere. When the characteristic length is of the order $1\mu m$, the magnitudes of both are comparable and rotational effects will then be manifested in the NMR diffusion experiment.

S4 - Rotational diffusion equations

The equations below describe the decay of PFGSE NMR resonances in cases where both Brownian rotational and translation motions are considered for single rods of length $L$ and spheres of radius $r$, in the limit of unrestricted rotational diffusion $^7$.

\[
\text{Sphere} \quad S_i = S_0 \frac{\sin^2(\alpha r)}{\alpha^2 r^2} e^{-D_T \alpha^2 \Delta} \\
\text{Rods} \quad S_i = S_0 \frac{\cos(\alpha L) - 1 + \alpha L}{\frac{\alpha^2 L}{4}} \frac{\sin(t)}{t} e^{-D_T \alpha^2 \Delta}
\]

Models for $D_T$ and $D_R$ are those described in figure S1. The origin of the variance of $D_{eff}$ with $\Delta$ is in the pre-exponential factor. Hence the gradient of the plot is given by the pre-exponential factor and does not depend on the model used for $D_T$. Empirically, the length distributions of amyloid fibrils measured using AFM/TEM take the form of log-normal distributions (Gaussian-like but skewed towards the greater probabilities of finding longer fibrils). The normalised log-normal distribution, defined as:

\[
C(L) = \frac{1}{a_1 \sqrt{2\pi} L} \exp \left[ -\frac{(\ln(L/a_0))^2}{2a_1^2} \right]
\]
The maximum of this distribution is given by $L_{max} = a_0 e^{-a_1^2}$, and the full width half maximum is $2 \sinh(a_1 \sqrt{\ln 4}) L_{max}$. The modified equation to estimate the NMR signal from a species within a distribution for a given value of $(G, \delta, \Delta)$ is given by:

$$\ln \frac{S_i}{S_0} = \frac{\sum_{L=0}^{\infty} C(L) S_i(L)}{\sum_{L=0}^{\infty} C(L) \lim_{G \to 0} S_i(L)}$$

(4)

With a model for $D_T$, the NMR signal intensity for a given $a_1$ and $a_0$ can be calculated. By comparing calculated NMR intensities with experimental data, we arrive at the fitted distributions shown in figure 2C in the main manuscript.

References