Microsecond Rotational Dynamics of Actin: Spectroscopic Detection and Theoretical Simulation

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We have investigated the microsecond rotational dynamics of F-actin with transient phosphorescence anisotropy (TPA) spectroscopy, and analyzed the data to determine the relative contributions from rigid-body rotations and from intrafilament bending and twisting, using a theoretical model developed for DNA dynamics by Schurr and co-workers. The fits of the data to the model were constrained by independently determining the orientation of the dye’s absorption dipole (by transient absorption anisotropy, TAA) and the actin filament length distribution (by electron microscopy). We conclude that (1) the Schurr theory enables calculation of the torsional flexibility of actin independent of any contribution from rigid body rotations of the whole filament, (2) the TPA decays cannot be explained by rigid-body or bending rotations, but reflect primarily twisting motions within actin filaments, and (3) the dynamic properties of actin filaments are best ascribed to a continuous elasticity. This analysis establishes a firm methodological foundation for future studies of the effects of perturbations of the dynamics of actin on its functional properties.

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Introduction

Understanding the molecular mechanism of actomyosin-based muscle and non-muscle motility requires knowledge of the structure and dynamics of actin. The present study is focused on the rotational dynamics of actin filaments in solution in the microsecond time range. This time range is particularly interesting, since it covers mechanical transients in muscle fibers (Brenner, 1991) and motions of myosin heads bound to actin in solution (Berger et al., 1989) and in myofibrils (Berger & Thomas, 1993, 1994).

The first observations of microsecond rotational motion in actin came from saturation transfer EPR (ST-EPR) studies of spin-labeled actin (Thomas et al., 1979), which reported an effective correlation time of about 100 µs, and cooperative restriction of actin dynamics by myosin heads. Subsequent studies of the transient phosphorescence anisotropy (TPA) and transient absorption anisotropy (TAA) of actin labeled with eosin iodoacetamide (EoIA) confirmed that actin is rotationally mobile in the submillisecond time range, with a correlation time of about 250 µs (Mihashi et al., 1982). Yoshimura et al. (1984) analyzed TAA decays of eosin iodoacetamide (EoIA) labeled actin using a formalism developed by Barkley & Zimm (1979) to describe the torsional dynamics of DNA, and concluded that the motion of F-actin could be modeled by torsional twisting within a flexible rod. Sawyer et al. (1988) analyzed TPA decays of erythrosin iodoacetamide (ErIA) labeled F-actin using a formalism developed by Broersma (1960) and concluded that the motion of F-actin could be modeled as the rotation of rigid rods.

The main goal of the present research is to resolve this discrepancy by determining whether the microsecond dynamics of F-actin involves overall
(rigid-body) rotation, intrafilament bending, intrafilament torsion, or a combination of the three. This question is crucial for studies of the relationship between the structure of actin and its function in various motile processes: while overall rotational motions of filaments may be important for cytoskeletal dynamics, only intrafilament motions can be important for the molecular mechanism of actin-myosin interactions. In the present study, the rotational motions of actin were studied by TPA and TAA of actin labeled with erythrosin iodacetamide at Cys374. A particular advantage of using this dye is that the spectroscopic properties of ErIA F-actin have been well characterized, and ErIA labeling does not have a significant effect on the functional properties of actin (Prochniewicz et al., 1993; Ludescher & Liu, 1993; Ng & Ludescher, 1994).

Correlation of the present anisotropy decay results with previous results from electron microscopy and electron paramagnetic resonance allowed us to eliminate certain models for the microsecond rotational dynamics of actin; e.g. axial filament bending and isotropic wobble of actin protomers. We were thus able to focus our analysis on models in which the microsecond rotational motion occurs only azimuthally, about the long actin filament axis, using a formula expressing the anisotropy decay of flexible polymers in terms of the azimuthal displacement of elementary rods connected by elastic springs, originally developed by Schurr and co-workers to analyze DNA torsional dynamics (Allison & Schurr, 1979; Schurr, 1984; referred to as Schurr theory). This formula allows a more complete description of rotational motion than the previously used Barkley-Zimm and Broersma approaches, and has two important features. First, it separates the contributions of overall (rigid-body) rotation and intrafilament twisting to the anisotropy decay, making it possible to isolate intrafilament twisting. Second, the formula depends on structural parameters of the actin filament such as filament length and transition dipole orientation, making it possible to improve the resolution and reliability of the analysis by incorporating independently determined values for parameters. The use of this analytical method, coupled with the direct electron microscopic determination of actin filament length distribution and the measurement of both TPA and TAA on the same samples, greatly reduces the ambiguity of data interpretation and clarifies the microsecond rotational dynamics of actin.

Results

Model-independent analysis of phosphorescence anisotropy decay of ErIA F-actin

Phosphorescence anisotropy decay of ErIA-labeled F-actin, best fitted to a sum of \( n = 3 \) exponential terms and a constant according to equation (3) is defined by three correlation times \( \Phi_i \), three amplitudes \( A_i = r_i / r_0 \) and the values of initial (\( r_0 \)) and final (\( r_\infty \)) anisotropy (Table 1). The longest correlation time for rotational motion in actin, about 266 \( \mu s \), is within the 270 \( \mu s \) predominant lifetime of actin-bound ErIA, obtained by fitting the decay of total phosphorescence emission to the sum of three exponential terms, equation (3).

The phosphorescence anisotropy decay was unaffected by varying the total actin concentration from 0.06 to 2 mg/ml. The increase in the total protein concentration was achieved by the addition of unlabeled actin to a constant concentration of labeled actin (0.06 mg/ml actin, corresponding to 1 \( \mu M \) dye), which prevented optical artifacts due to an increase in the optical density of the sample.

Wobbling motions in actin

The value of the initial phosphorescence anisotropy \( r_0 = r(t = 0) \) of actin-bound ErIA, 0.12 (±0.003) (SEM, \( n = 14 \)) was significantly lower than the value obtained for ErIA completely immobilized in PMMA resin, where \( r(t = 0) = r_0 = 0.205 \), most likely due to the fast, submicrosecond motion of the dye relative to actin (Ludescher & Liu, 1993) and/or fast motion of the dye-binding region of actin (Ikkai et al., 1979; Miki & Kouyama, 1994). The angular amplitude of these submicrosecond time scale motions can be calculated using the “wobble in a cone” model, equation (5). Substituting \( r(t_{\max}) = r_0 = r(t = 0) \) and \( r(t_{\min}) = r_0 \) gives \( \theta^o = 31.7 (±1.3)^o \) (mean ± SEM, \( n = 22 \)), similar to the value of 25.9\(^o\) obtained directly from nanosecond fluorescence anisotropy decay of pyrene iodoacetamide attached to Cys374 (Sasaki et al., 1994).

We then considered the possibility that the microsecond TPA decay is also due to isotropic wobble of the actin-bound ErIA. Substituting \( r_0 = r(t = 0) \) and \( r_\infty \) for \( r(t_{\min}) \) and \( r(t_{\max}) \) in equ-

| Table 1. Phosphorescence anisotropy decay parameters for ErIA-labeled F-actin |
|----------------------|-----------------|-----------------|------------------|---------------|-------------------|-----------------|-----------------|
| \( \Phi_1 (\mu s) \) | \( \Phi_2 (\mu s) \) | \( \Phi_3 (\mu s) \) | \( A_1 \) | \( A_2 \) | \( A_3 \) | \( r_0 \) | \( r_\infty \) |
| 5.82 ± 0.46         | 37.81 ± 1.67    | 266.43 ± 18.97  | 0.178 ± 0.013   | 0.284 ± 0.007 | 0.320 ± 0.015   | 0.127 ± 0.003   | 0.037 ± 0.008   |

ErIA-labeled F-actin (0.06 mg/ml), phalloidin-stabilized, in 50 mM KCl, 20 mM Tris (pH 8.0), 0.1 mM CaCl\(_2\), 0.2 mM ATP, 25°C. Non-linear least-squares fit of the anisotropy decay \( r(t) \) to a sum of exponential terms, equation (3), with normalized amplitudes \( A_i = r_i / r_0 \) and rotational correlation times \( \Phi_i \), presented as mean ± SEM, \( n = 14 \) data files from seven independently prepared samples.
Table 2. The effect of the exchange of the tightly bound ADP into BrADP in ErIA F-actin on phosphorescence anisotropy decay

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\Phi_1$ (µs)</th>
<th>$\Phi_2$ (µs)</th>
<th>$\Phi_3$ (µs)</th>
<th>$r_0$</th>
<th>$r_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-ADP</td>
<td>4.81 ± 0.89</td>
<td>43.44 ± 3.51</td>
<td>370.37 ± 31.61</td>
<td>0.098 ± 0.002</td>
<td>0.203 ± 0.037</td>
</tr>
<tr>
<td>F-BrADP</td>
<td>3.71 ± 0.79</td>
<td>42.28 ± 4.16</td>
<td>341.17 ± 39.79</td>
<td>0.089 ± 0.003</td>
<td>0.019 ± 0.003</td>
</tr>
</tbody>
</table>

Non-linear least-squares fit of the anisotropy decay to a sum of three exponential terms, equation (3), with rotational correlation times $\Phi_i$. The numbers represent mean ± SEM, $n = 8$ from four independent samples of ADP F-actin and $n = 6$ from three independent samples of BrADP F-actin. The exchange of the nucleotide was carried out following the procedure described by Orlova & Egelman (1993). The protein concentration was adjusted to 0.5 mg/ml, phalloidin was not added, and the temperature was 25°C.

Bending motions of actin filament

Both light scattering (Fujime et al., 1984) and fluorescence microscopy (Yanagida et al., 1984) have indicated that thermal bending motions of actin filaments occur on the millisecond time scale, much slower than the motions measured in the present study. To clarify this point further, we estimated the contribution of thermal bending to the phosphorescence anisotropy decay by comparing data from actins of different bending rigidity, produced by exchange of BrADP for tightly bound ADP in ErIA F-actin. While the exchange caused an 80% decrease in bending rigidity, as measured by electron microscopy (Orlova & Egelman, 1993), it had no significant effect on the correlation times and final anisotropy; the slight difference in the initial anisotropy likely reflects differences in fast, submicrosecond time scale motions (Table 2). Thus we conclude that the millisecond time scale thermal bending motions of the filaments do not make a significant contribution to the microsecond time scale dynamics of F-actin, consistent with previous theoretical calculations (Yoshimura et al., 1984).

Modeling of the microsecond dynamics of actin as torsional (azimuthal) rotational motions of a segmented flexible cylinder (Schurr theory)

Length distribution of labeled actin filaments

Figure 1 shows a typical result of an electron microscopic measurement of the length distribution of ErIA-labeled F-actin by electron microscopy. The bell-shaped profile of the length distribution and number average filament length $L_n = 4.2 ± 0.8$ µm (mean ± SD, $n = 4$), were not affected by labeling. These results are similar to results recently reported (Janmey et al., 1986; Burlacu et al., 1992), but differ from the exponential form reported earlier (Kawamura & Maruyama, 1970). The length distribution was independently measured by fluorescence microscopy, yielding $L_n = 5.2$ µm, essentially the same as obtained by electron microscopy. This result confirms the earlier report (Burlacu et al., 1992) that actin filament preparation for electron microscopy, i.e. staining
Rotational Dynamics of Actin

Figure 2. TAA decay of phalloidin-stabilized, ErIA-labeled F-actin (0.5 mg/ml, 25°C); (a) data superimposed on the fit to \( n = 3 \) exponential terms, equation (3); (b) residual.

Figure 3. Fit of TPA decay of phalloidin-stabilized, ErIA-labeled F-actin (0.06 mg/ml, 25°C) to the rigid-body rotational model (equation (14)); (a) data superimposed on fit; (b) residual. The fit function included only rotation about the long filament axis (spinning). The diffusion coefficients, \( D_\parallel \), were calculated using the length distribution shown in Figure 1, where \( L_o = 4.2 \mu m \). Constant parameters of the fit were \( u_a = 29.9 (\pm 2.5)° \) (SD), and \( d = 34.5° \); adjustable parameters were \( u_e \) and \( k \).

and grid attachment, has no significant effect on the length of F-actin.

**Determination of the orientation of the absorption dipole of actin-bound ErIA**

Figure 2 shows a typical TAA decay of ErIA F-actin. Fitting the decay to the sum of three exponential terms (equation (3)), \( (n = 3) \) yielded correlation times of \( \Phi_1 = 2.21 (\pm 0.19) \mu s, \Phi_2 = 27.31 (\pm 5.00) \mu s, \) and \( \Phi_3 = 192.17 (\pm 29.29) \mu s \) (mean ± SEM, \( n = 14 \)), similar to correlation times of the TPA decay (Table 1). The value of the amplitude reduction factor \( k \) (0.73 (± 0.02), mean ± SEM, \( n = 42 \)) was similar to the value obtained from TPA (\( k = 0.61 (\pm 0.02) \), mean ± SEM, \( n = 23 \)). This similarity of the fit result indicates that the two methods report the same motions.

Substituting the fitted values of the initial \( r_i \) and final \( r_f \) anisotropy into equation (10), gives \( \theta_e = 30.2 (\pm 0.6)° \) (mean ± SEM, \( n = 14 \)). This value was fixed in subsequent analysis of TPA data. The orientation of the emission dipole, \( \theta_e \), and the interdipole angle, \( \psi_{ia} \), were allowed to vary in the fit procedure (equation (11)).

**Overall motions of actin filaments (rigid-body rotation)**

Simulation of the TPA decay as a result of rigid-body rotation, as described by equation (14), produced the same results regardless of whether or not \( D_\perp \) was included in equation (14), indicating that end-over-end tumbling of filaments is too slow to contribute to microsecond anisotropy decay. Therefore, all subsequent fits assumed that rotational motions of filaments are limited to spinning about the long axis, i.e. \( D_\perp = 0 \). The large discrepancy between the simulated and experimental decays, as indicated by the residuals and high \( \chi^2 \) (164) shows that the TPA decay is not exclusively caused by overall rotation of actin filaments (Figure 3).

When all fit parameters were permitted to float, the fit did improve, but the residuals and still high \( \chi^2 \) (22) showed that the model cannot fit the decay; furthermore, the fit found \( \theta_e = 87.1° \), far outside the range allowed by experimental error, based on the TAA measurements (25.6 to 33.3°).

We independently tested the possibility of rigid-body rotation of actin filaments by examining the effect of increasing viscosity, through addition of glycerol. Analysis in terms of a sum of three exponential terms (equation (3)) showed that a sixfold increase in viscosity, from 1 cp in buffer to 6 cp in 50% glycerol, did not have a significant effect on correlation times: the longest correlation time \( \Phi_3 \) increased at most 59%, while the shorter correlation times, \( \Phi_1 \) and \( \Phi_2 \), were unchanged or even slightly decreased. This result supports our conclusion that overall rotations of the filaments do not contribute to TPA. The analysis also indicated changes in the amplitudes of rotational motions, suggesting
glycerol-induced changes in the length and/or structure of the filaments.

**Torsional twisting within the actin filament**

We fitted actin TPA to equation (15), which assumes both rigid body rotation and torsional twist, and where \( C_n(t) \) is given by equation (16) (Allison & Schurr, 1979; Schurr, 1984). In the first series of fits we assumed that the elementary rods have the height of one actin protomer (\( h = 55 \) Å) and the diameter of the filament (\( a = 45 \) Å; Holmes et al., 1990). The fit is good, as shown by the small residual (Figure 4); moreover, the fitted value of \( \kappa = 0.58 \) (mean \( \pm \) SD, \( n = 4 \)) agrees well with \( \kappa = r_0/r_0^0 = 0.61 \) (mean \( \pm \) SD, \( n = 23 \)), found by fitting the data to a sum of three exponentials, equation (3). Analysis of four data files gave \( \alpha = 4.0 \ (\pm 1.0) \times 10^{-12} \) dyn cm (mean \( \pm \) SD).

The effect of uncertainty in the experimental \( \psi_0 \) (determined from TAA, Figure 2) on the results was evaluated by fitting one TPA decay with two constant values of \( \psi_0 , 25.6 \) and 33.4°, which were the minimum and maximum values obtained in five independent TAA experiments. A satisfactory fit was obtained in both cases, and the two values found for \( \alpha , 2.7 \times 10^{-12} \) dyn cm and \( 2.3 \times 10^{-12} \) dyn cm, agree within 15%.

The effects of the height \( h \) and radius \( a \) of the elementary rod were examined by fitting a data file at several different values of \( h \) and \( a \). Parameters \( \psi_0 \) and \( \theta_0 \) were fixed to the values \( h = 55 \) Å, \( a = 45 \) Å obtained from the fit (Figure 4); parameters \( \alpha \) and \( \kappa \) were permitted to vary. The results are summarized in Figure 5.

The model gave satisfactory fits of the anisotropy decay for heights of the elementary rod ranging from half of the protomer to one crossover of the filament, provided the radius of the elementary rod was the same as the radius of the actin double helix (Figure 5(a), (b), (c)). While changes in the height of the elementary rod from 55 to 27 to 356 Å resulted in changes of \( \alpha \) from \( 2.5 \times 10^{-12} \) dyn cm to \( 5.0 \times 10^{-12} \) dyn cm to \( 0.4 \times 10^{-12} \) dyn cm, respectively, the product, \( \alpha h = C \), the torsional rigidity of the actin filament, remained constant at \( 1.4 \times 10^{-18} \) dyn cm². This indicates that the model cannot uniquely define the size of the rotating unit within the actin filament; the most likely conclusion is that the model describes elastic properties of actin in terms of the torsional rigidity \( C \), i.e. the model regards actin as a continuous elastic rod. The decrease in the quality of the fits when the radius of the filament was decreased (Figure 5(d), (e))
suggested that the microsecond time scale rotational motions involve the double-helical structure rather than motions in each of the two strands.

Subsequent fitting of the anisotropy decay to the Intermediate Zone Formula (equation (17)), which ignores the contribution from rigid body rotations, resulted in \( \alpha = 3.0 \pm 0.2 \times 10^{-12} \) dyn cm (SD, \( n = 4 \)), which is essentially the same as the fit to equation (15). This result is consistent with the conclusion that rigid body rotation does not contribute significantly to the TPA of F-actin.

**The effect of phalloidin on the TPA of ErIA F-actin**

Phalloidin binding slightly but significantly increased the rates of TPA decay (Figure 6), and the results of fitting the decays to equation (15) show that this difference is due to a 40% decrease in the spring constant, from \( \alpha = 12.0 \pm 1 \times 10^{-12} \) dyn cm to \( \alpha = 6.6 \pm 0.6 \times 10^{-12} \) dyn cm, which translates to increased torsional flexibility. Such increase does not contradict a phalloidin-induced twofold decrease in the bending flexibility (Isambert et al., 1995), since we have shown that slow thermal bending motions have no effect on the microsecond time scale dynamics of F-actin (Table 2).

**Comparison with previous results**

Comparison of the present results of simulation of torsional dynamics of actin using the Schurr theory with the previously published results of analysis of TAA of EoIA-labeled actin (Yoshimura et al., 1984), who used the Barkley-Zimm formalism (Barkley & Zimm, 1979), requires:

1. **Labeling of actin with the same phosphorescent dye**, i.e. EoIA. We have confirmed that the correlation times and amplitudes of the decay of actin labeled with EoIA: \( \Phi_1 = 1.98 \pm 0.13 \), \( \Phi_2 = 39.05 \pm 3.5 \), \( \Phi_3 = 305.5 \pm 3.5 \), \( A_1 = 0.434 \pm 0.019 \), \( A_2 = 0.133 \pm 0.007 \) and \( A_3 = 0.224 \pm 0.022 \) are essentially the same as those of ErIA-labeled actin (Table 1). Decreasing the fraction of labeled monomers to 20% by copolymerization of ErIA-labeled and unlabeled actin, which made the extent of labeling of actin similar to that of Yoshimura et al. (1984), had no effect on the decay.

2. **Fitting the anisotropy decays to similar formalisms.** Like Yoshimura et al. (1984), we fitted the TPA to the Barkley-Zimm expression, which assumes that the absorption and emission dipoles are parallel. The resulting spring constant \( \alpha = 5.5 \times 10^{-12} \) dyn cm is not significantly different from \( \alpha = 4.0 \pm 1 \times 10^{-12} \) dyn cm, obtained using Schurr theory (equation (15)).

3. **Comparison of the values of torsional rigidities**: the values of \( \alpha \) obtained in this work for various heights of an elementary rod give \( C = 1.4 \times 10^{-18} \) dyn cm², which is comparable to the previously reported value of \( 2 \times 10^{-18} \) dyn cm² (Yoshimura et al., 1984).

**Discussion**

**Rigid body rotation cannot explain the microsecond dynamics of F-actin**

The first important result of this work is that rigid-body rotation of actin filaments in solution cannot explain the observed microsecond time scale anisotropy decay: the predicted overall rotations are much slower than the experimentally detected motions (Figure 3). The discrepancy with previous analysis of overall filament rotation (Sawyer et al., 1988) is most likely related to different assumptions used in simulations. The analysis by Sawyer et al. (1988) was based on assumed, rather than measured, filament lengths and did not take into account the orientation of the probe transition dipoles. In contrast, our conclusion is based on incorporating the structural parameters, lengths of filaments \( L_n \) and probe absorption dipole orientation \( \theta_n \), in the fitting procedure: we have demonstrated that changes in the length distribution of filaments (Zhang et al., 1994) as well as changes in the orientation of transition dipoles significantly change the time course of the calculated decay and so will significantly affect the conclusion.

In view of the importance of \( L_n \) and \( \theta_n \) for the analysis, it is important to assess the reliability of these independently determined parameters: (1) \( L_n \). The possibility that monomers and short oligomers, which were difficult to visualize at 5000× magnification and thus were not accounted for in the length distribution, contribute to the anisotropy decay seems unlikely for the following reasons.

(a) **Stabilization of actin filaments by phalloidin not only inhibits depolymerization during dilution but also decreases the critical concentration of actin** (Estes et al., 1981); in this particular case, it would...
Intrafilament torsional twisting

Filament length heterogeneity is a major confounding factor in studies of actin dynamics, both from structural perturbations and interactions with actin-binding proteins. The most important feature of the Schurr theory is that it clearly distinguishes contributions to the anisotropy decay from overall and intrafilament motions, and so enables determination of the torsional flexibility of the filament. This is particularly important for comparison of filaments with different average length and it enabled us to demonstrate that chemical modifications of actin, which result in inhibition of sliding movement but also shorten the length of actin filament, are accompanied by changes in torsional flexibility of actin filaments (Prochniewicz et al., 1995).

Another important feature of the Schurr theory is that it allows us to address the molecular interpretation of flexibility: whether we detect motions of defined structural elements in F-actin filament or its continuous elasticity. We addressed this question by changing the dimensions of the elementary rod in the fits. Since the height of the elementary rod, whether one protomer, half a protomer or one helix crossover, had no effect on the quality of the fit (Figure 5(a), (b), (c)), we conclude that the model cannot define the twisting element in the structure of F-actin. This conclusion disagrees with the earlier proposal (Yoshimura et al., 1984) that torsion originates from azimuthal rotations of single protomers, which was supported by a good agreement between the amplitude of twist motions (obtained by expressing the continuous elasticity of the Barkley-Zimm formalism as an elasticity per protomer rise in the actin filament) and the r.m.s. azimuthal disorder of protomers in the filament (determined from electron microscopy by Egelman et al., 1982). If the torsional rigidity of actin detected by TPA reflects azimuthal motion of protomers, then the observed decrease in the extent of angular disorder in the actin filaments upon phalloidin binding (Orlova & Egelman, 1993) should be reflected in an increase in torsional rigidity. However, we did not observe such an increase: on the contrary, phalloidin binding decreased the microsecond torsional rigidity of actin. A previous report (Yoshimura et al., 1984) that phalloidin had no effect on the microsecond dynamics of actin was most probably due to lower precision of the data. Since phalloidin interacts with three neighbouring monomers in actin (Lorenz et al., 1993), the increase in torsional flexibility of actin by phalloidin might be considered surprising. However, as explained above, the TPA data suggest a continuous flexibility, which may originate from the superposition of all motions in the protomers and thus be relatively unaffected by stabilization of some of the intermonomer contacts.

The torsional rigidity of actin found in this work, C = 1.4 × 10⁻¹⁸ dyn cm², is about one order of magnitude lower than the theoretically predicted...
2.6 \times 10^{-17} \text{ dyn cm}^2 \text{ (ben-Avraham & Tirion, 1995), but the discrepancy is not unexpected. First, the theoretical analysis ignores solvent effects, which affect calculated rates of motion. Second, the theory assumes that torsional rigidity of F-actin, defined as the resistance of the filament to distortions caused by external torque, resides only in the interprotomer bonds, the protomers being completely rigid. However, experimental measurements on a dye bound to one specific site in the protein reflect a superposition of motions occurring in actin, including motions within the protomers (Tirion & ben-Avraham, 1993).

We explored the possibility that motions occur independently in both strands of the actin filament by setting the diameter of the elementary rod to be the diameter of one protomer regarded as a sphere. Independent motion of the strands of the actin filament relative to each other were proposed in Censullo & Cheung’s (1993) lateral slipping model as an alternative explanation of the angular disorder in actin, and were also predicted by theoretical calculations (ben-Avraham & Tirion, 1995). The poorer fit quality (Figure 5(d), (e)) indicates that, in this model, such motions are not likely explanations of the anisotropy decay. It may be that the model applied here is not adequate to describe slipping motion of strands, or, alternatively, that these motions can occur on a time scale different from that of the phosphorescence anisotropy measurement.

This study establishes a reliable methodology for measuring quantitatively the internal rotational dynamics of actin in the microsecond time scale, which are dominated by torsional twisting motions. This sets the stage for future studies correlating these motions with function.

Materials and Methods

Preparation of proteins

Actin was extracted from acetone powder from rabbit skeletal muscle with cold water (0 to 4°C) with or without 5 mM Tris, 0.2 mM ATP and 0.1 mM CaCl$_2$ (pH 8.0), polymerized with 30 mM KCl for one hour at 25°C, and ultracentrifuged for one hour at 200,000 g (Strzelecka-Golaszewska et al., 1980). The pellet was suspended and depolymerized (to G-actin) in 0.3 mM ATP, 0.2 mM CaCl$_2$, 5 mM bicarbonate (pH 8.0), and clarified by 30 minutes centrifugation at 150,000 g. The protein ran as a single band in SDS-PAGE. Actin concentration was determined by measurement of absorbance at 290 nm, using ε (1 mg/ml) = 0.630. The molar concentration of actin was calculated assuming a molecular mass of 42,500.

Labeling of actin at Cys374

Labeling was always performed using freshly prepared actin. G-actin (2 mg/ml, corresponding to 48 μM), was polymerized to F-actin for one hour at 25°C in 0.1 M KCl, 0.2 mM CaCl$_2$, 0.3 mM ATP and 20 mM bicarbonate buffer (pH 8.0), and then incubated for three hours in this buffer at 25°C with 432 μM of erythrosin iodoacetamide (ErIA) or eosin iodoacetamide (EoIA), freshly dissolved in DMF; the final concentration of DMF in actin was less than 1%. The reaction was terminated by addition of 10 mM DTT, and the samples were dialyzed overnight against 20 mM Tris, 50 mM KCl, 0.1 mM ATP and 0.1 mM CaCl$_2$ (pH 8.0). The remaining, unreacted dye was removed by ultracentrifugation for one hour at 200,000 g, depolymerization of the pellet in 5 mM Tris, 0.3 mM ATP and 0.1 mM CaCl$_2$ (pH 8.0), and chromatography on a Sephadex G-25 column equilibrated with the same buffer. The labeled actin was immediately polymerized in F-buffer (50 mM KCl, 20 mM Tris, 0.2 mM ATP, 0.1 mM CaCl$_2$ (pH 8.0)) for two to three hours at 25°C and stabilized by adding phalloidin at a 1:1 molar ratio; this procedure ensured that actin was entirely in F-form during spectroscopy. We confirmed that 99% of the polymerized ErIA actin used in the spectroscopic measurements sedimented during one hour centrifugation at 200,000 g. ErIA F-actin was stored on ice and used for experiments within three days.

The extent of labeling, determined by measuring the absorbance at 539 nm, was 0.83 (±0.09) (SD, n = 14) mol ErIA per mol of actin, and 0.75 (±0.07) (SD, n = 7) mol EoIA per mol of actin, assuming molar extinction coefficients at 539 nm of 83,000 M$^{-1}$ cm$^{-1}$ for actin-bound ErIA (Sawyer et al., 1988) and 95,000 M$^{-1}$ cm$^{-1}$ for actin-bound EoIA (Eads & Thomas, 1984). The concentration of labeled actin was measured by the Bradford assay (Bradford, 1976), with unlabeled actin as a standard.

Specificity of F-actin labeling at Cys374 by ErIA was confirmed by competition with N-ethyl maleimide (NEM). Actin (48 μM, in 50 mM KCl, 10 mM Tris, 0.2 mM ATP, 0.1 mM CaCl$_2$ (pH 8.0)) was incubated for 40 to 60 minutes at 23°C with 220 to 480 μM NEM, which has previously been shown to label Cys374 quite specifically under these conditions (Lusty & Fasold, 1969; Elzinga & Collins, 1975). When this NEM-preblocked actin was then incubated with ErIA, the ErIA reaction was completely inhibited, showing that ErIA reacts specifically with Cys374.

Preparation of samples for spectroscopic experiments

TPA and TAA experiments were done on ErIA F-actin in F-buffer, at 25°C. To prevent triplet-state quenching and photobleaching during spectroscopy, oxygen was enzymatically removed (Eads & Thomas, 1984) by adding glucose oxidase (220 μg/ml), catalase (36 μg/ml), and glucose (45 μg/ml), and gently blowing argon above the sample surface. Under these conditions the excited-state lifetime of the actin-bound ErIA reached a constant value after five minutes incubation at 25°C. Control experiments confirmed that purification of actin by gel filtration on Sephadex G-200 prior to labeling had no effect on the spectroscopic signal.

Electron microscopy

Samples of phosphorescent actin, prepared for spectroscopic experiments, were diluted to 0.02 mg/ml in
F-buffer, applied to glow-discharged, collodion- and carbon-film-coated copper grids, stained by 1% uranyl acetate, and observed with a JEOL 100 CX electron microscope at an accelerating voltage of 80 kV. Photographs were taken at 5000 × magnification, and negatives were printed on 8 inch × 10 inch Ilford photographic paper. The distribution of filament lengths was determined by tracing the prints with a digitizing tablet.

Spectroscopic experiments

Microsecond rotational motions of phosphorescent-labeled actin were detected essentially as described previously for myosin by both transient phosphorescence anisotropy (TPA; Ludescher & Thomas, 1988) and transient absorption anisotropy (TAA; Ludescher et al., 1987). In both experiments, the sample of labeled actin was excited by a vertically polarized pulse from a XeCl-pumped dye laser at 540 nm, using 5 mM coumarin 548 in ethanol, operating at a repetition rate of 100 to 200 Hz.

In TPA experiments (Ludescher & Thomas, 1988), the time-resolved phosphorescence anisotropy was calculated from:

\[ r(t) = \frac{I_v - GI_{th}}{I_v + 2GI_{th}} \]  

(1)

where \( I_v(t) \) and \( I_h(t) \) are the vertically and horizontally polarized components of the emission signal, obtained by signal averaging the time-dependent phosphorescence emission decays, detected at 90° with a single detector and a polaroid sheet polarizer that alternates between the two orientations every 1000 laser pulses. \( G \) is an instrumental correction factor, determined by performing the experiment with a solution of free dye and adjusting \( G \) to give an anisotropy value of zero, the theoretical value for a rapidly and freely tumbling chromophore. A typical experiment involved 20 cycles of 2000 pulses (1000 in each orientation), at a laser repetition rate of 100 Hz; data acquisition time was therefore about seven minutes.

TAA experiments were carried out in a similar manner, except that the signal detected was the absorbance of a continuous 100 μW probe beam at 514.5 nm, provided by an argon ion laser. The only significant modification from our previous TAA instrument (Ludescher et al., 1987) was that prisms were placed before and after the sample, so that, while the two laser beams were precisely colinear at the sample, the transmitted probe beam could be detected without interference from the intense excitation pulse. The change in absorbance \( \Delta A(t) \) is defined as \( \log(I_0/I(t)) \), where \( I(t) \) and \( I_0 \) are the transmitted intensities with and without the excitation pulse. Excitation was vertically polarized, and the polarization of the probe beam was adjusted by rotation of a half-wave plate to give equal vertical and horizontal components of polarization incident on the sample. The absorption anisotropy \( r_A(t) \) was calculated from:

\[ r_A(t) = \frac{\Delta A_v - \Delta A_h}{\Delta A_v + 2\Delta A_h} \]  

(2)

where \( \Delta A_v \) and \( \Delta A_h \) correspond to absorption with the polarizer set vertically and horizontally.

Anisotropy data analysis

Model-independent fit: sum of exponentials

The phosphorescence and absorption anisotropy decays were fitted to a sum of exponential terms and a constant:

\[ r(t) = \sum_{i=1}^{n} r_i e^{-t/\tau_i} + r_c \]  

(3)

where \( \tau_i \) are the rotational correlation times (inversely related to the rates, or diffusion coefficients, of rotation), \( r_i \) are corresponding amplitudes (related to the angular amplitudes of rotation, molar fractions of rotating species and orientation of the observable transition dipole), and \( r_c \) is the final (residual) anisotropy.

The observed initial anisotropy as fit to equation (3), is defined as \( r_0 = r(0) = \Sigma r_i + r_c \). The theoretical maximum value of \( r_0 \) is \( r_0^2 = 0.4 (3 \cos^2 \delta - 1)/2 \), where \( \delta \) is the angle between the excited (absorption) and observed (emission) transition dipoles of the probe (Figure 7). In practice, the observed (best-fit) initial anisotropy \( r_0 \) can be lower than \( r_0^2 \) by an amplitude reduction factor \( \kappa \leq 1 \):

\[ r(t = 0) = r_0 = \kappa r_0^2 = 0.4 \kappa P_2 (\cos \delta) \]  

(4)

Variation of the laser intensity and sample concentration assured that neither optical saturation nor light scattering affected the data, so \( \kappa \) was due entirely to the angular amplitude of submicrosecond probe rotations. \( r_0^2 \) (and so, by equation (4), \( \delta \)) was determined directly from the measured anisotropy of the dye immobilized in the polymethylmethacrylate (PMMA) block, assuming that the angle \( \delta \) between the probe’s transition moments is independent of the probe environment. The PMMA block was prepared as follows: 10 μl of 10 mM ErIA dissolved in DMF was added to 9.99 ml of methylmethacrylate, followed by the addition of 10 mg of 2-methylpropanenitrile powder (Vazo-64, DuPont Chemical Co., Wilmington, Delaware).
The solution was incubated for one hour at 80°C, then cooled overnight at room temperature until totally polymerized. The polymerized solid was machined into a 1 cm x 1 cm x 4 cm block. For the immobilized erythrosin standards used in the present study, the time-independent anisotropy $r_0$ of 0.205 corresponded to $\delta = 34.8^\circ$.

**Wobble-in-a-cone model**

For the rotational motion involving isotropic wobble of the observed transition dipole of the probe in a cone with half angle $\theta_0$, $\theta_0$, is given by (Kinosita et al., 1977):

$$ r(t_{\text{max}}) = \frac{1}{2} \cos \theta_0 (1 + \cos \theta_0) $$

where $r(t_{\text{max}})$ and $r(t_{\text{min}})$ are the final and initial anisotropy, respectively. The amplitudes of motions in the submicrosecond or microsecond time windows were estimated by substituting either $r_0/r_0' (\equiv k)$ or $r_\perp/r_0$ for the left side of equation (5).

**Segmented flexible cylinder: general model**

According to the model (Allison & Schurr, 1979; Schurr, 1984) the filament is regarded as a randomly labeled linear array of cylindrical elementary rods with a local coordinate system embedded in each rod; the orientation of absorption ($\mu_\alpha$) and emission ($\mu_\beta$) dipoles of the rod-bound probe in the local coordinate system is shown in Figure 7. As a consequence of the combined global and internal rotational brownian motions of the whole filament, each particular rod (and its bound dye) undergoes in time $t$ mean-squared displacements about its body-fixed $x$, $y$, and $z$ axes.

The general anisotropy is expressed as (adapted from Allison & Schurr, 1979; Schurr, 1984):

$$ r(t) = r(t_{\text{max}}) = \frac{1}{2} \cos \theta_0 (1 + \cos \theta_0) $$

where $k$ is the amplitude reduction factor defined above (equation (4)).

For the rigidly bound dye, the amplitudes $A_n$ are defined by:

$$ A_0 = \frac{1}{10} (3 \cos^2 \theta_0 - 1) (3 \cos^2 \theta_0 - 1) $$

$$ A_1 = \frac{6}{10} \sin \theta_0 \cos \theta_0 \cos \psi_{\theta_0} \sin \theta_0 \cos \theta_0 $$

$$ A_2 = \frac{3}{10} \sin^2 \theta_0 \cos 2\psi_{\theta_0} \sin^2 \theta_0 $$

The angle $\theta_0$ was calculated from the initial $r_0$ and final $r_\perp$ values of the TAA anisotropy decay (Kinosita et al., 1977):

$$ \theta_0 = \arccos \left(\frac{1}{2} \pm \frac{1}{2} \sqrt{\frac{r_\perp}{r_0}} \right) $$

The angles determining the orientation of the two transition dipoles of the probe (Figure 7) are constrained by the relation

$$ \cos \delta = \cos \theta_0 \cos \theta_0 + \sin \theta_0 \cos \psi_{\theta_0} \sin \theta_0 $$

**Rigid-body rotational model**

For completely rigid filaments of length $l_i$, equation (6) can be expressed as:

$$ r_i(t) = \frac{k}{N} \exp[-t(D_i + 5D_i)] + A_2 \exp[-t(4D_i + 2D_i)] $$

where $a$ is the radius of the rod, $\eta$ is the solvent viscosity, and $D_i$ and $D_\perp$ are the diffusion coefficients around $z$ and $x$, respectively.

For a population of filaments having a broad length distribution, the observed anisotropy is the sum of the contributions from filaments within each particular length group $l_i$, weighted by the fraction of labeled protomers in the filaments belonging to the group:

$$ r(t) = \sum_{i=1}^{n} r_i(t) \beta_i $$

$$ \beta_i = \frac{k_i}{\sum_{i=1}^{n} k_i} $$

where $k_i$ is the number of filaments with length $l_i$.

**Torsional twisting model**

For flexible filaments that do not perform bending motions, $F_\theta(t) = 1$, and the general anisotropy becomes:

$$ r(t) = \frac{n^{\frac{1}{2}} K_\theta T l_i}{(N_i + 1) \gamma} \left(\frac{N_i - 1}{N_i + 1} \right) \times \sum_{m=1}^{N_i+1} \exp[-n^2 \sum_{r=1}^{N_i+1} d^2_r Q^2_r(1 - c^{t/u})]

where the expression for the twisting correlation function $C_{\gamma}(t)$ (Schurr, 1984) is given by:

$$ C_{\gamma}(t) = \frac{n^{\frac{1}{2}} K_\theta T l_i}{\sqrt{(N_i + 1) \gamma}} \times \sum_{m=1}^{N_i+1} \exp[-n^2 \sum_{r=1}^{N_i+1} d^2_r Q^2_r(1 - c^{t/u})]

\gamma = \frac{\gamma_\alpha}{2} \sin^2 \left(\frac{\pi}{2} \left(\frac{s - 1}{2} \right)\right)

\tau_{\alpha} = \frac{\gamma_\alpha}{2} \sin^2 \left(\frac{3}{2} \pi \left(\frac{s - 1}{2} \right)\right)

\frac{1}{\sqrt{(N_i + 1)}} \cos \left(\frac{m - 1}{2} \left(\frac{s - 1}{2} \right)\right)

\delta_{\alpha} = \frac{1}{\sqrt{(N_i + 1)}} \delta_{\alpha}

\alpha$ is the torsional spring constant between rods and $\gamma$ is the friction factor for rotation of a rod about its z axis. For a perfectly cylindrical rod, $\gamma = 4\pi \eta a^2 h$, where $h$ and $a$ are its height and radius, respectively, and $\eta$ is the solvent viscosity.
The approximation of equation (15) that ignores the contribution from rigid-body rotations to the anisotropy decay, the so-called Intermediate Time Zone (ITZ) formula, is given by:

\[ r(t) = \kappa \left[ A_0 + A_1 \exp\left( -\frac{t}{4K_B T} \right) + A_2 \exp\left( -\frac{t}{\Phi} \right) \right] \]

(17)

\[ \Phi = \frac{(\pi a)^2 \gamma}{4 R_e \tau^2} \]

(18)

If we assume that the absorption and emission dipoles of the probe are parallel, equation (17) has the same functional form as the formula derived by Barkley & Zimm (1979), which was used previously for analysis of TAA of EoIA-labeled actin (Yoshimura et al., 1984).

**Methods of simulation and fitting**

For models three to five above, the anisotropy decay was calculated from the indicated expressions (equations (14) and (15)), and the indicated parameters were varied to minimize the sum of squared differences between the actual and simulated data, \( \chi^2 \). In most cases, this was done using a Pascal program on a microcomputer, but in the case of equation (15) this was carried out with a Cray-YMP C90 supercomputer (Cray Research, Eagan, MN) at the Minnesota Supercomputer Institute. A bounded modified Levenberg-Marquardt algorithm using a finite-difference Jacobian (IMSL function BCLSF, IMSL, Inc.) was used to solve the non-linear least-squares problem. The filament length distribution, experimental anisotropy decay, and model parameters were input to the fit program.

**Reagents**

All phosphorescent dyes, erythrosin iodoacetamide (EriA) and eosin iodoacetamide (EoIA), were purchased from Molecular Probes (Eugene, OR) and stored at -20°C. ATP, 8-bromoadenosine-5′-triphosphate (BrATP) and phallolidin were obtained from Sigma (St Louis, MO). All other reagents were of reagent grade.

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**References**


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