DYNAMIC LIGHT-SCATTERING EVIDENCE FOR THE FLEXIBILITY OF NATIVE MUSCLE THIN FILAMENTS

JAY NEWMAN AND FRANCIS D. CARLSON, *Department of Biophysics, Johns Hopkins University, Baltimore, Maryland 21218 U.S.A.*

ABSTRACT We have obtained clear evidence for the flexibility of native scallop adductor thin filaments by studying the temperature and ionic strength dependence of the average decay constants obtained from intensity fluctuation spectroscopic (IFS) measurements. The low-angle (10–25°), average decay constants obtained from time autocorrelation functions of scattered light were independent of concentration (0.08–1.3 mg/ml), scaled with the ratio of temperature to solvent viscosity, T/η, over a range of 4–45°C, and yielded a value for the translational diffusion coefficient of $D_T^{5°C} = (1.24 \pm 0.06) \times 10^{-8}$ cm^2/s. From this value and the Broersma relation for rigid rods, we find an average filament length of 1.06 ± 0.06 μm. Quantitative sodium dodecyl sulfate polyacrylamide gel electrophoresis showed that at high temperatures (> 35°C) or in 0.6 M NaCl, tropomyosin completely dissociates from native thin filaments. Decay constants from high-angle (60–150°C) IFS temperature dependence measurements do not scale with T/η and hence do not show the temperature dependence expected for rigid rods. The differences are not due to any change in length distribution of filaments with temperature or to the free tropomyosin in solution, but are attributed to nonrigid motions of the filaments. Similar experiments on samples in high- and low-salt solvents gave results consistent with this interpretation.

INTRODUCTION

The protein actin, in its fibrous form, has long been known to be involved in muscular contraction, and recently it has been shown that actin is involved in an essential way in many biological contractile and motile processes. A knowledge of the dynamic properties of native thin filaments and their components is needed to assess their possible active role in contractile mechanisms.

Dynamic light-scattering measurements on F-actin and its complex with tropomyosin have been reported and interpreted in terms of filament flexibility (Fujime, 1970; Fujime and Ishiwata, 1971; Ishiwata and Fujime, 1971). Subsequent reports on F-actin (Carlson and Fraser, 1974; Fraser et al., 1975), in which the time autocorrelation functions of the scattered light rather than the power spectral densities were measured, produced experimental results in disagreement with the earlier work and left open the question of filament flexibility. Maeda and Fujime (1977) have reported new data on F-actin solutions, obtained using a digital autocorrelator, which essentially agree with those of Carlson and co-workers. They have pointed out some of the complexities of analyzing data from such highly polydisperse samples.

A preliminary report of this work was presented at the 1978 meeting of the Biophysical Society, and appeared in abstract form (*Biophys. J.* 1978. **21**:104*a*.).

Dr. Newman's present address is Department of Physics, Union College, Schenectady, N.Y. 12308.

We have considered the question of thin filament flexibility by studying a more monodisperse preparation, native scallop thin filaments. Although a full analysis of our dynamic light-scattering data cannot be completed in the absence of an appropriate theory, we have obtained clear evidence for nonrigid behavior (flexibility) of these native thin filaments in solution.

EXPERIMENTAL PROCEDURES

Sample Preparation and Characterization

Native thin filaments were prepared from the adductor muscles of deep sea scallops (*Placopecten magellanicus;* obtained live from the Marine Biological Laboratory, Woods Hole, Mass.), essentially by the procedures of Szent-Györgyi et al. (1971) and Lehman and Szent-Györgyi (1975). Some of the samples were then passed through a Bio-gel A150M column (Bio-Rad Laboratories, Richmond, Calif.; 2 × 30 cm; standard solvent: 50 mM NaCl, 5 mM NaPi, 2 mM $MgCl_2$, 3 mM NaN_3, pH 7.0; rate, 4 ml/h; 4°C) to obtain a sharper length distribution of filaments.

The samples were routinely characterized by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis following a modified procedure of Davies and Stark (1970). Total contamination by proteins other than actin and tropomyosin, as determined by optical density scans of the gels stained with Coomasie blue, was usually <1% with no, or only a trace amount of, myosin present on heavily overloaded gels. The densitometric ratio of tropomyosin to actin was 0.32 ± 0.05 (SD of 10 preparations). Protein concentrations were measured using a modified Lowry method (Hartree, 1972). As another method of characterizing our samples, we measured the length distributions of thin filaments from three preparations by electron microscopy (JEOL Analytical Instruments, Cranford, N.J., model JEM-T8), using negative staining with 1% uranyl acetate.

Tropomyosin Binding Studies

Centrifuge studies of the binding of tropomyosin to actin filaments were performed at various temperatures and ionic strengths. Native thin filament preparations were adjusted to the solvent conditions of the experiment and thermally equilibrated in the centrifuge chamber before centrifugation at 105,000 *g* for 3 h (40,000 rpm in a Ti 65 rotor; solvent volume 5–10 ml in No. 339574 tubes, Beckman Instruments, Inc., Fullerton, Calif.). The gently washed pellets were resuspended in the same solvent, and they and the combined supernatant/wash solutions were analyzed for protein content by quantitative SDS gel electrophoresis and by protein concentration determinations.

Intensity Fluctuation Spectroscopy

The light-scattering apparatus and method of data collection have been described previously (Carlson et al., 1972; Goll and Stock, 1977). Measurements were made using the 488-nm argon ion laser line (fundamental transverse mode, TEM_{00}) with input power ranging from 1 to 100 mW; the correlation times obtained were independent of power level in this range. The laser beam was focused with its ~80-μm-diam waist at the center of the scattering cell. An immersion oil-to-glass beam stop arrangement at the exit window of the thermostatted bath (regulation: ±0.05°C) eliminated significant back reflections. Without the beam stop, data obtained above 90° scattering angle were distorted by forward scattering from the back reflected beam. Calibration of the optical system using 0.1-μm polystyrene latex spheres indicated that reliable prescaled intensity fluctuation spectroscopic (IFS) measurements could be obtained over an angular range of 10–150° and that there was no convection in the thermostatted sample cell over a range of temperatures from 2 to 45°C.

Samples were prepared for the light-scattering experiments by centrifugation at 35,000 *g* for 2 h in stoppered tubes (SS-34 rotor, 5–10-ml samples in the same tubes as given above). The samples were then transferred into optical cells in a positive pressure dust-free box; the cells and pipettes were cleaned inside a special acetone refluxing apparatus. Solvents prepared in this way produced <2% of the

scattering intensity of a similarly prepared 1 mg/ml solution of thin filaments at an angle of 20°, and this level fell sharply at higher scattering angles.

All measurements were repeated 20 or more times and the duration of a single experiment was adjusted to be long enough to obtain an unbiased estimate of the coherence time (Saleh and Cardoza, 1973). The prescaled intensity autocorrelation functions were normalized to the independently determined background levels (corrected for prescale error, Jakeman et al., 1972) and analyzed using the cumulant method of Koppel (1972) which determines both an average decay constant, $\bar{\Gamma}$, and a normalized second cumulant, $\mu_2/\bar{\Gamma}^2$, reflecting the polydispersity of the sample. Normalized raw data were plotted to check the scaling laws described below (see Fig. 1). Several sets of field autocorrelation

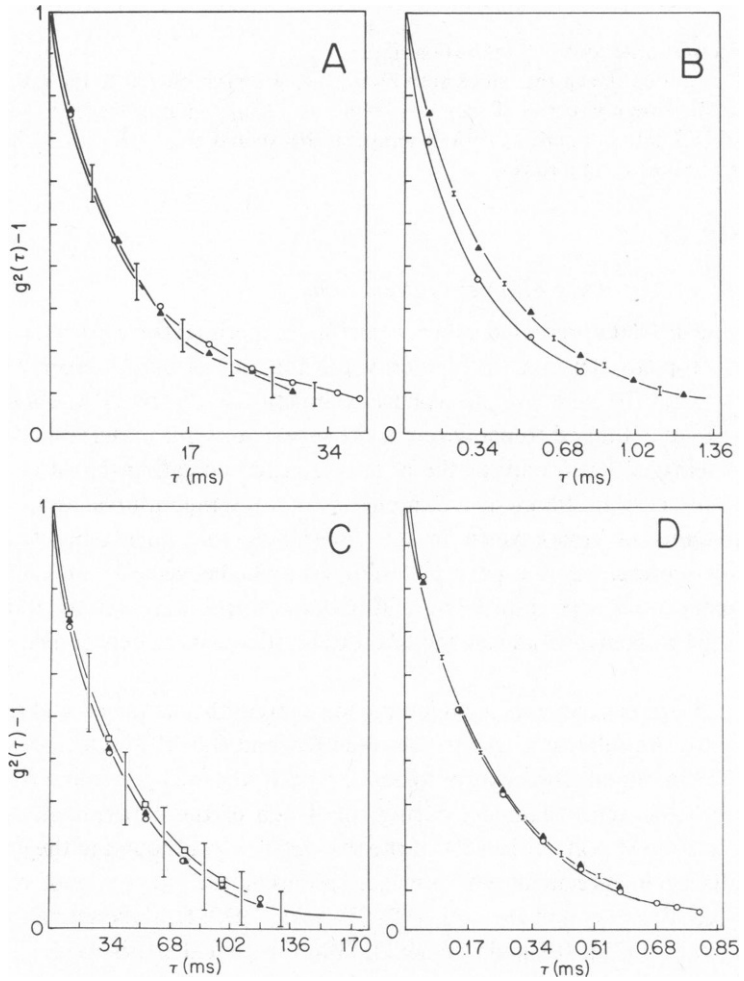

FIGURE 1 (A and B) Normalized autocorrelation function data for a thin filament solution at 1.26 mg/ml in our standard solvent + 2 mM ATP. Measurements were performed first at 5.5°C (▲); the sample was then heated to 44°C (○). The time axes have been scaled by T/η to 5°C (see Eq. 1 in text). Measurements are at scattering angles of 25° in A and 120° in B. Only a few of the 60 data points are shown for each curve with representative error bars indicated. Vertical bars on the low-temperature curves indicate the standard deviations of the data points from 25 separate experiments, and they apply to the other curve(s) as well. (C and D) Same for a 0.27 mg/ml solution of fd virus in 150 mM KCl, 15 mM NaPi, pH7.0. The data are for 4.8°C (▲), 22.4°C (□), and 40.4°C (○). Scattering angles are 12° in C and 120° in D. For clarity, in D the 22.4°C data are not plotted since they fall on the 5.5°C data points.

data (obtained by means of the Seigert relation) were also fit to the noninteracting rigid-rod model of Pecora (1964):

$$g^{(1)}(\tau) = \sum_{\ell \text{ even}} B_\ell(qL) \exp[-(D_T q^2 + \ell(\ell+1)D_R)\tau], \quad (1)$$

where $g^{(1)}$ is the normalized field autocorrelation function, B_ℓ is a known function, q is the scattering vector, L is the rod length, and D_T and D_R are the translational and rotational diffusion coefficients of the rod. We also fit our data to the model of Fujime (1970; see also Fujime and Maruyama, 1973):

$$g^{(1)}(\tau) = A \exp(-D_T q^2 \tau) + B \exp[-(D_T q^2 + 1/\tau_1)\tau], \quad (2)$$

where τ_1 is the lowest order bending relaxation time.

From Eq. 1, it can be shown that since both D_T and D_R are proportional to the ratio of the absolute temperature to the solvent viscosity, T/η, $g^{(1)}(\tau)$ scales as $(T/\eta)\tau$. Hence, the average decay constants computed from IFS data obtained at various temperatures should also scale with T/η if the scatterers are stable, noninteracting, rigid rods.

RESULTS

Tropomyosin-Actin Filament Interactions

To interpret the IFS data presented below, centrifuge experiments were performed to measure the binding of tropomyosin to actin filaments as a function of both temperature and solvent ionic strength. Thin filament samples spun at temperatures below 20°C showed only a few percent of the total actin and tropomyosin in the supernate. This material had the same ratio of the two proteins as that found for the pellet material, indicating that it represented short fragments of intact thin filaments. Samples spun at temperatures above 25°C showed increasing amounts of tropomyosin in the supernates with increasing temperature. No tropomyosin was present in the pellets of samples spun between 35 and 45°C. When the resuspended pellet and supernate from a 40°C experiment were mixed and spun at 4°C, essentially all the tropomyosin was bound to the actin filaments as determined by quantitative SDS gels.

Similar centrifuge experiments in which the ionic strength was varied and the temperature held at 4°C gave the following results. Between 0.3 and 0.6 M NaCl there was a gradual decrease in the binding of tropomyosin to actin. At 0.6 M NaCl the tropomyosin completely dissociated from the actin filaments during the 4–5 h of the experiment. After overnight dialysis back to 50 mM NaCl over 95% of the tropomyosin was bound to the actin filaments.

These results are in agreement with previous studies on the interaction of synthetic F-actin with tropomyosin (Tanaka and Oosawa, 1971; Tanaka, 1972; Drabikowski and Nowak, 1968; Spudich and Watt, 1971). It should be noted, however, that the IFS-results presented below indicate that the binding of tropomyosin to actin filaments, after its removal from native thin filaments by either heating or increased ionic strength, is not identical to that in the native filament even though the tropomyosin to actin densitometric ratio of 0.32 is preserved.

Dynamic Light Scattering

TRANSLATIONAL DIFFUSION Thin filament samples that were maintained at low temperatures (2–5°C) in our standard solvent gave reproducible IFS results over periods of 3–5 d. In all experiments, the amplitude of the correlation function, $g^{(1)}(0)$, was the same,

within an experimental uncertainty of ±0.03, as that obtained with polystyrene latex spheres. This indicates the absence of any heterodyning effects from stray light or large "dust" particles and the absence of any gel-like properties as has been observed with F-actin-heavy meromyosin samples (Carlson and Fraser, 1974). The depolarized component of the light scattered from a 1 mg/ml thin filament solution was <0.05% that of the polarized component in the angular range of 15–150°, indicating the absence of significant multiple scattering. The data generally fit a single exponential decay moderately well as indicated by $\mu_2/\bar{\Gamma}^2$ values ranging from 0.2 to 0.4 for 20 different preparations. These values did not significantly vary with angle from 10 to 150° or with channel time (range: 1/60–1/10 correlation time), and the experimental data decayed to the independently determined background, indicating the absence of very large aggregates as reported for F-actin by Maeda and Fujime (1977); their $\mu_2/\bar{\Gamma}^2$ values for F-actin ranged from 2 to 12 and strongly depended upon q^2 as well as channel time.

Our results are qualitatively similar to those obtained with F-actin solutions (Carlson and Fraser, 1974; Maeda and Fujime, 1977) in that $\bar{\Gamma}$ approaches zero at forward scattering angles and that at higher angles (≥30°) the data show an increasing positive slope (see Fig. 2 A). The low-angle (≤25°; this angular range was not studied in previous light-scattering

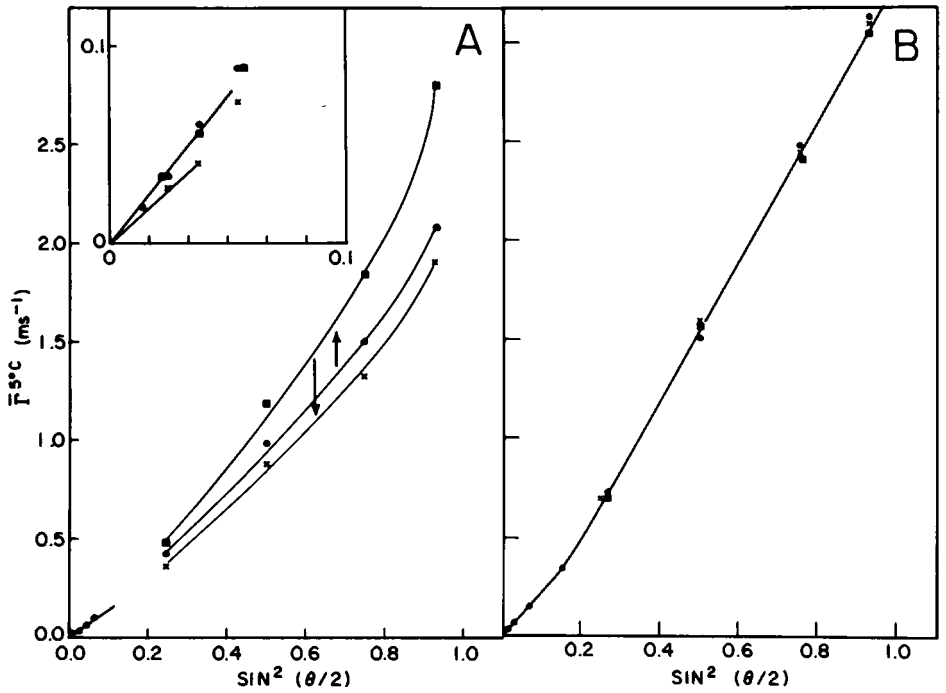

FIGURE 2 (A) Average decay constants computed for the thin filament solution of Fig. 1 as a function of $\sin^2(\theta/2)$. Measurements were performed first at 5.5°C (●); the sample was then heated to 44°C (■), and the angular measurements were repeated; this was followed by another set of measurements after cooling to 4.8°C (X). All the data has been scaled by T/η to 5°C (see Eq. 1 in the text). The insert is an enlargement of the low-angle data. (B) Same for the fd solution of Fig. 1. The results at three different temperatures 5°C (●), 22°C (■), and 40°C (X) are plotted after scaling the decay constants by T/η to 5°C; values at the lower angles superimpose.

studies of F-actin) $\bar{\Gamma}$ values are proportional to $\sin^2(\theta/2)$ ($\propto q^2$) and are independent of protein concentration over a range of 0.08–1.3 mg/ml indicating little if any interaction between thin filaments. The $\bar{\Gamma}$ values at all angles reproduced within ±2% when one sample was repeatedly studied over several days; however, sample-to-sample variations in the $\bar{\Gamma}$ values were larger, ±5% at the lower angles and ±10% at angles of 90° or greater. From the average slope of the low-angle results from 92 measurements, we obtain a value for the translational diffusion coefficient $D_T^{5°C} = \bar{\Gamma}/q^2 = (1.24 \pm 0.06) \times 10^{-8}$ cm^2/s, where the uncertainty is three times the standard error of the mean. Using the Broersma equation relating D_T to the length and diameter of a rigid rod (Broersma, 1960; Newman et al., 1977) and assuming a filament diameter of 90 Å, we obtain an average length of 1.06 ± 0.06 μm for the equivalent rigid rod. This value for the filament length is in agreement with a value of 1.01 ± 0.09 μm SD obtained by Millman and Bennett (1976) by electron microscopy of longitudinal sections of scallop adductor muscles.

TEMPERATURE DEPENDENCE Thin filament samples in our standard solvent immersed in a water bath at 45°C were monitored by IFS measurements at a 25° scattering angle repeated every 2 min. Over a period of 40 min, the average diffusion coefficient increased by a factor of 8, indicating that the filaments were depolymerizing. In the presence of 2 mM ATP, however, the average diffusion coefficient remained unchanged, indicating that the actin filaments remain intact despite the fact that tropomyosin dissociates from the actin filament at 45°C (see above). The dissociated tropomyosin contributes little to the intensity of the scattered beam and would not be expected to significantly contribute to the correlation functions obtained (see below). Figs. 1 A, B, and 2 A show the results of more detailed temperature dependence IFS measurements. Consider first the low-angle results shown in Fig. 1 A and the insert in Fig. 2 A. Normalized raw data and the derived $\bar{\Gamma}$ values overlap within error the corresponding values at 5.5 and 44°C when the time axis and $\bar{\Gamma}$ values are each scaled by T/η (see Eq. 1). At low scattering angles only the center of mass motion (translational diffusion) can be detected in the polarized IFS data. This means that although the tropomyosin had been completely dissociated from the actin filaments, the length distribution of the filaments had not significantly altered. After the sample is cooled back to 5°C, the decay constants reduce in value by approximately one-third, indicating substantial aggregation of the filaments (Fig. 2 A). It follows, therefore, that upon recooling the tropomyosin does not bind in a completely reversible manner even though it has been shown above to rebind to the actin filaments in the same total amount as in the native filaments. This suggests the cross-linking of actin filaments by tropomyosin to some extent. Since the length distribution of the filaments changed upon cooling back to 5°C, the high-angle data in this case would be expected to, and indeed does, show reduced $\bar{\Gamma}$ values as well.

The higher-angle, normalized raw data and the computed decay constants do not scale with T/η and hence do not show the temperature dependence which theory predicts for a distribution of noninteracting rigid rods (see Eq. 1 and below). The decay constants obtained at 40–45°C at high angles were consistently larger (faster decay) than those measured at 5°C after scaling by T/η. To determine whether the difference could be due to the free tropomyosin in solution, the average scattered intensities and intensity correlation functions were measured on the supernate from a native thin filament preparation after centrifugation at 40°C. The supernate contained all the tropomyosin from the thin filaments and some small

actin filament fragments. The scattered intensities were only ~1% that of the initial thin filament preparation, and the correlation times were smaller by a factor of ~14 in approximate agreement with the calculated ratio of 16.3 based on the Broersma relations and a length and diameter of 400 and 20 Å, respectively, for tropomyosin (Caspar et al., 1969). With these scattering properties the material in the supernate could not produce the temperature dependence observed when present together with the actin filaments. In addition, thin filament samples that had not been prepared using the Bio-gel column, and which had a larger number of fragments as determined by electron microscopy, still showed the same high-angle temperature dependence.

To verify the theory of T/η scaling of IFS results, we studied a sample of fd virus (kindly provided by Dr. Loren A. Day; the virus is a rod with a length and diameter of ~0.9 μm and 90 Å, respectively) as a prototype of a rigid-rod system with comparable dimensions to thin filaments. A previous study (Newman et al., 1977) concluded that fd virus behaves hydrodynamically as a rigid rod in solution, although they did not measure the temperature

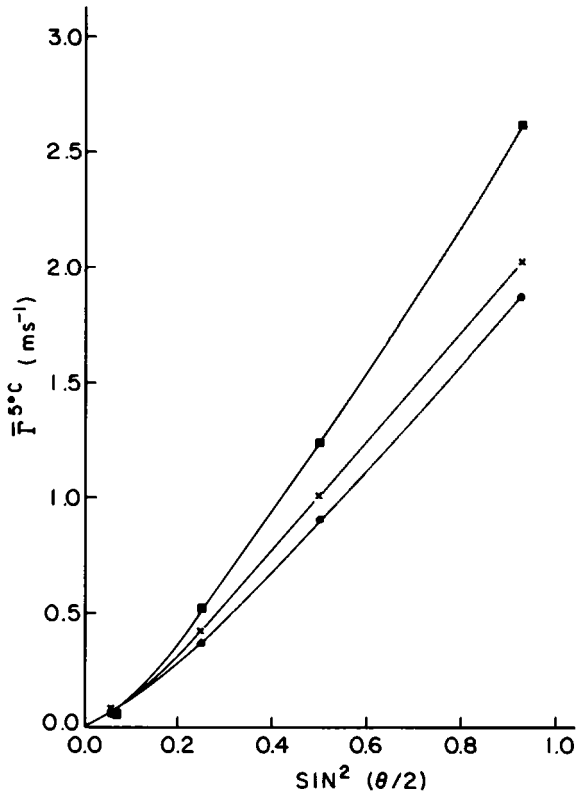

FIGURE 3 Effect of 0.6 M NaCl on the thin filament IFS decay constants obtained at 5°C. Measurements were obtained for an initial 2.3-mg/ml preparation in our standard solvent (●). An equal volume of 1.2 M NaCl was then slowly added directly to the light-scattering cell in a "dust-free" box while gently stirring using a small Teflon (DuPont de Nemours & Co., Inc., Wilmington, Del.) magnetic stirrer (■). Correlation functions were then recorded from low to high to low scattering angles to insure that the results did not change over the ~1 h required for measurements. A 1:1 dilution of the starting material into the standard solvent served as a control of the concentration dependence of the decay constants (X).

dependence of the IFS decay constants. Figs. 1 C, D, and 2 B present the results of such a set of temperature-dependence measurements. These results show clearly that the decay constants do indeed scale with T/η over the same range of temperatures employed in the thin filament experiments. The translational diffusion coefficient obtained from the low-angle - $\bar{\Gamma}/q^2$ values is $(2.6 \pm 0.1) \times 10^{-8}$ cm^2/s in agreement with the previous result (Newman et al., 1977).

IONIC STRENGTH DEPENDENCE As an independent method of obtaining IFS data on actin filaments that have been obtained from thin filaments by the complete dissociation of tropomyosin, we studied thin filament samples in 0.6 M NaCl. The centrifuge binding study results (see above) indicated that in 0.6 M NaCl the tropomyosin completely dissociates from the actin filaments which depolymerize slightly over several hours. The IFS measurements were done rapidly at only four angles to avoid changes in the actin filament length distribution with time. The absence of significant change was verified by repeated low-angle measurements at the start and end of the experiments. Addition of millimolar concentrations of ATP did not stabilize the filaments in 0.6 M salt. The results are shown in Fig. 3. At 30° scattering angle the $\bar{\Gamma}$ values all agree, indicating the same length distribution of filaments in each case. However, at higher angles the angular dependence of $\bar{\Gamma}$ from samples in 0.6 M NaCl at 5°C is similar to that observed for the samples heated to 40–45°C (see Fig. 2 A) and does not agree with the low-salt results. Controls similar to those of the temperature-dependence studies were run to determine if the tropomyosin and actin filaments present at 0.6 M NaCl could produce the $\bar{\Gamma}$ values observed. Again this was not found to be possible. Consequently, we believe the observed $\bar{\Gamma}$ values reflect only the motions of the actin filaments themselves.

DISCUSSION

The absence of T/η scaling for native thin filaments cannot be explained by any of the usual artifacts of light-scattering experiments such as aggregation, stray light, or "dust" since these would decrease $\bar{\Gamma}$ and decrease the amplitude of the correlation function. We have also eliminated other potential explanations such as multiple scattering (negligible depolarized scattering), changes in length distribution of the filaments (consistent low-angle T/η scaling), interactions between filaments ($\bar{\Gamma}$ independent of concentration over 16-fold range), thermal convection (not observed with polystyrene latex sphere calibrations), and the scattering of free tropomyosin (too little and $\bar{\Gamma}$ too large). Therefore we believe we are justified in attributing the absence of T/η scaling to some flexibility of the filaments.

If we accept this explanation, we can obtain estimates for the root mean square amplitude, ξ, and the time scale of the bending motions of the filaments. To detect a component of our measured correlation functions due to flexibility, ξ must be of the order of several hundred Angstroms ($\sim 1/q$ at high angles so that $q\xi \sim 1$). If ξ were much smaller, the light-scattering technique would not detect this component and if much larger, the D_T value obtained from low-angle data would be expected to be significantly increased at higher temperatures, which was not observed. The time scale associated with these bending motions must necessarily be comparable to our experimental time scales at high scattering angles (approximately hundreds of microseconds, i.e., $\sim 1/\bar{\Gamma}$), otherwise we would not have detected a flexibility component.

The decay constants from high scattering angle IFS data at 5°C are larger for thin filament

solutions in 0.6 M NaCl than in 50 mM NaCl (see Fig. 3). This increase approximates that observed in the temperature-dependence studies (see Fig. 2 A). Together these results suggest that the removal of tropomyosin is responsible for the increased $\bar{\Gamma}$, which we have attributed to a change in the filament flexibility. Studies now in progress are directed toward establishing this interpretation more conclusively.

The early dynamic light-scattering studies of F-actin solutions by Fujime and co-workers (Fujime, 1970; Fujime and Ishiwata, 1971; Ishiwata and Fujime, 1971) were analyzed using a modified form of their model (Eq. 2) in which the first term was omitted ($A = 0$). We both expected and confirmed that Eq. 2 does not give an adequate fit to our data. Our expectations were based on the fact that Fujime's model neglects contributions to the correlation function from rotational diffusion. Since a 1-μm $\times$ 90-Å rod has a rotational correlation time ($1/6D_R$) of ~17 ms and translational diffusion correlation times ($1/D_T q^2$) longer or comparable to this for angles of 30° or less, this approximation is not valid in our case. We also question whether it would be valid for the exponential length distribution of F-actin. The modified form also does not apply, as pointed out by Carlson and Fraser (1974), since the magnitude of B approaches 0 at forward scattering angles, whereas A approaches 1 (see Fig. 3 in Fujime and Maruyama, 1973). In fact, fits to Eq. 2 (with either A a fitting parameter or $A = 0$) for data at various scattering angles gave poor fits with nonconsistent values for D_T and τ_1.

There are several theories of the dynamic light-scattering properties of Rouse-Zimm chain molecules (Lee et al., 1977; de Gennes, 1976; Edwards and Grant, 1973; Doi, 1975). Although thin filaments and F-actin are definitely not such molecules, there have been recent references to the possible applicability of these theories to long, semirigid, rod-like macromolecules (Lee et al., 1977; Gethner and Gaskin, 1978). None of these theories offers an explanation for our observation that T/η scaling is not obeyed. Nevertheless, we tried to fit our data for $g^{(1)}(\tau)$ to the sum of two exponentials with each decay constant scaling with q^2 according to the model of Lee et al (1977). The decay constants and relative amplitudes obtained from such fits did not show the angular dependence predicted by the theory. Entanglement theories (de Gennes, 1976) predict that the cooperative diffusion coefficient (measured by dynamic light scattering) of semidilute solutions should scale as $c^{3/4}$, where c is the concentration. This predicts an ~eightfold increase in $\bar{\Gamma}$ over the range of concentrations we studied (0.08–1.3 mg/ml). We observed no change of $\bar{\Gamma}$ over this range. In addition, de Gennes predicts that at low scattering angles there should be an elastic component in the IFS data. We observed no evidence for such a component. There seems, therefore, to be no experimental basis for the application of any of these theories to dynamic light scattering from thin filaments and F-actin.

There is as yet no complete theory for analyzing our data in detail. However, it is possible to compare the observed average decay constants to those obtained from computer-generated data for $g^{(1)}$ based on the rigid-rod model (Eq. 1). Computer-generated data for a 1-μm $\times$ 90-Å rigid rod give a plot of $\bar{\Gamma}$ vs. $\sin^2(\theta/2)$ that is roughly linear for low scattering angles ($\leq$25°) with a transition to another linear region of greater slope at the higher angles (>45°). This result is similar to our data for fd virus but the high-angle thin filament data are clearly nonlinear (see Fig. 2 A). To simulate the effects of polydispersity, computer-generated data for mixtures of discrete length rods were analyzed. We found that although polydispersity can produce high-angle curvature in a plot such as Fig. 1 A, reasonable mixtures of rod lengths

yield $\bar{\Gamma}$ values very different from our experimental ones. Our choices for discrete length distributions were based on rough estimates obtained from electron microscopy data. We do not consider these length distribution estimates to reliably reflect the distributions in solution because of the fragmentation and aggregation that occur when native thin filaments are prepared for microscopy. (Recall also that our high-angle, experimental $\bar{\Gamma}$ values were insensitive to the presence of rod fragments in precolumn preparations.) Thus, the high-angle results at any particular temperature are another indication of nonrigid motion of the thin filaments.

We thank Mrs. F. Liu for skilled technical assistance, Dr. L. A. Day for providing the fd virus sample, and Dr. A. G. Szent-Györgyi for advice in the preparation of scallop thin filaments.

This work was supported by U.S. Public Health Service grants AM-12803 and AM-16315 to Dr. Carlson and by a postdoctoral fellowship from the Muscular Dystrophy Association of America to Dr. Newman.

Received for publication 14 October 1978 and in revised form 3 August 1979.

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