LETTER TO THE EDITOR

The Flexibility of Isolated Limulus Thick Filaments in Relaxed and Activated States

Dear Sir:

In previous work, we found that the average line width $\bar{\Gamma}$, of the photoelectron count autocorrelation function of light, scattered from a suspension of isolated thick filaments from *Limulus* striated muscle, increased dramatically when exposed to calcium ions. The increase in $\bar{\Gamma}$ values was shown to be related to cross-bridge motions that required ATP hydrolysis. However, the possibility that an increase in the flexibility of the thick filaments could also be responsible for a component of the increase in $\bar{\Gamma}$ values was not eliminated. Using dark-field light microscopy, we have found that the flexibility of the thick filaments actually decreases after they have been activated and shortened in the presence of calcium ions. This observation further strengthens our contention that the increase in $\bar{\Gamma}$ values is due primarily to cyclic motion of the cross bridges in the presence of calcium ions and ATP.

We measured the photoelectron count autocorrelation function of light scattered from a suspension of thick filaments isolated from the striated muscle of *Limulus*. The average line width, $\bar{\Gamma}$, increased dramatically following the addition of calcium ions. At a scattering angle of 120°, for instance, the $\bar{\Gamma}$ value increases on average ~2.2-fold (Fan et al., 1983*a*; Kubota et al., 1983). The increase of $\bar{\Gamma}$ values is reversible. *Limulus* striated muscle is both thin- and thick-filament regulated (Lehmann and Szent Gyorgyi, 1975; Sellers, 1981). We have shown that the increase in $\bar{\Gamma}$ values requires the hydrolysis of ATP and reflects cross-bridge motion following activation by calcium ions (Fan et al., 1983*b*, 1984, 1985). However, the possibility still existed that the increase in $\bar{\Gamma}$ values was partly due to an increase in the flexibility of the thick filaments when exposed to calcium ions.

Dark-field light microscopy has been used to visualize the image of objects with diameters <20 nm (Macnab and Koshland, 1974; Hotani, 1976; Macnab and Ornston, 1977; Kamiya et al., 1979). Later, dark-field light microscopy was used by Nagashima and Asakura (1980) to deduce the flexibility of actin filaments. We have used the same method to determine the flexibility of thick filaments from *Limulus* striated muscle, and have shown that their flexibility actually decreases upon activation with calcium ions and their subsequent shortening.

Thick filaments were isolated from the levators of the telson of *Limulus*, as described previously (Dewey et al., 1978, 1982, 1984; Kubota et al., 1983), except that the magnesium ion concentration of the solutions used was increased to 5 mM instead of 2mM. The isolated filaments were first dialyzed against a relaxing solution (100 mM KCl, 5 mM $MgCl_2$, 5 mM Tris, 0.1 mM EGTA, and 2 mM ATP at pH 7.2). We designate these filaments as relaxed. An aliquot of this filament suspension was then dialyzed against an activating solution (relaxing solution containing 5 mM $CaCl_2$ in addition). We call these filaments activated. The light-scattering results obtained with thick filaments, suspended in a medium containing either 2 or 5 mM $MgCl_2$, were practically the same. A microscopy (Carl Zeiss Inc., Thornwood, NY) with oil immersion dark-field condenser (N.A. 1.2/1.4), planapo 40 × oil immersion objective (N.A. 1.0), and arc lamp (model XB0 150; Xenon, Wilmington, MA) were used. The image was recorded on videotape with a high sensitivity television camera (model TV3M; Venus Scientific Inc., Farmingdale, NY) and a video-recorder (model VO-5800; Sony Corp., Long Island City, NY) and was monitored continuously on a video screen. The filament suspension, on a slide, was covered with a coverslip and sealed with grease. All measurements were done at room temperature, which was ~20°C. The contour length of the thick filaments was measured with a digitizer graphic calculator (model 1224; Numonics Corp., Lansdale, PA).

After the slides were mounted, they were searched for thick filaments using the television monitor. Groups of filaments of uniform length and brightness, displayed horizontally in the field, could be identified. Since their lengths were very close to the measured lengths of thick filaments in fixed and sectioned muscle with sarcomere lengths above 7.0 μm (Dewey et al., 1973), we believe that the images were of isolated thick filaments. Further confirmation was obtained by staining the suspension with fluorescein-labeled antibody against *Limulus* myosin heavy chain. The structures we identified as thick filaments, in fact, fluoresced intensely. For thick filament length measurement we chose only filaments whose entire length was in clear focus. The focal depth of the optical system we used was ~0.5 μm. Under such conditions, the maximum tilting angle of the filaments from a plane perpendicular to the direction of view would be $\lesssim \sin^{-1}(0.5/4.8) = 6°$ for the relaxed filaments with an average length of 4.8 μm, and $\sin^{-1}(0.5/3.3) = 9°$ for the activated filaments with an average length of 3.3 μm. The error in the measurements of the filament length was, respectively, <0.03 and 0.04 μm for the relaxed and the activated filaments.

The elastic modulus of bending, ϵ, was caluculated according to Harris and Hearst (1966):

$$\epsilon = \frac{3kT}{4\lambda}, \tag{1}$$

where k and T are the Boltzmann constant and the absolute temperature and λ is a parameter correlated with the flexibility. λ can be calculated with two equations given by Landau and Liftshitz (1958, Eqs. 148.6 and 148.7). The two equations permit us to calculate the λ with two different measured parameters. The equations are:

$$\bar{R}^2 = [2\lambda L - 1 - \exp(-2\lambda L)]/2\lambda^2 \tag{2}$$

and

$$\overline{\cos\theta\,(L)} = \exp(-\lambda L). \tag{3}$$

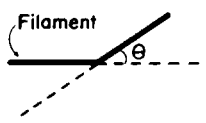

FIGURE 1 Illustrates the method of measurement of $\Theta(L)$.

TABLE I.
FLEXIBILITY PARAMETER (λ) AND ELASTIC MODULUS OF BENDING (ϵ) OF RELAXED, ACTIVATED, AND RE-RELAXED THICK FILAMENTS FROM *LIMULUS* STRIATED MUSCLE

Filament state	Filament length	Calculated with Eq. 2		Calculated with Eq. 3	
		λ	ϵ	λ	ϵ
	(μm)	*(μm^{-1})*	*(dyn $cm^2 \times 10^{17}$)*	*(μm^{-1})*	*(dyn $cm^2 \times 10^{17}$)*
Relaxed	4.84 ± 0.25	0.033 ± 0.007	9.2 ± 1.9	0.035 ± 0.008	8.7 ± 2.0
Activated	3.31 ± 0.29	0.023 ± 0.007	13.2 ± 2.8	0.022 ± 0.006	13.8 ± 3.4
Re-relaxed	3.20 ± 0.23	0.025 ± 0.005	12.1 ± 2.5	0.024 ± 0.005	12.6 ± 2.5

In Eq. 2, R and L are, respectively, the end-to-end distance and the contour length of the filaments, and in Eq. 3, $\theta(L)$ is the angle between the tangent at the two ends of the filaments as illustrated diagrammatically in Fig. 1.

Measurements were first made on relaxed filaments. The average value of the contour length was 4.84 ± 0.25 μm and that of the end-to-end length, 4.56 ± 0.38 μm. The contour length of filaments isolated in relaxing solution containing 5 mM $MgCl_2$, as we used here, was longer than that of filaments isolated in relaxing solution containing 2 mM $MgCl_2$. This difference has also been confirmed by electron microscopy of negatively stained filaments isolated under the two concentrations of $MgCl_2$ (Dewey et al., unpublished results). The λ value calculated according to Eq. 2 is 0.033 ± 0.007 μm^{-1}, and the elastic modulus of bending ϵ is (9.2 ± 1.9) × 10^{-17} dyn cm^2. The cos (L) equals 0.837 ± 0.026. The λ value calculated according to Eq. 3 is 0.035 ± 0.008 μm^{-1}. Accordingly, the elastic modulus of bending ϵ is (8.7 ± 2.0) × 10^{-17} dyn cm^2.

Measurements were then made on activated filaments. The average values of the contour length and the end-to-end length were, respectively, 3.31 ± 0.29 and 3.22 ± 0.30 μm. The percentage difference between the end-to-end distance and the contour length was smaller for the activated filaments. It was 3% for the activated filaments, as opposed to 6% for the relaxed filaments. The $\overline{\cos\theta\,(L)}$ equaled 0.93 ± 0.08, which was much larger than the value obtained for the filaments in relaxing solution. These facts alone quantitatively indicate that the activated filaments are less flexible. This was confirmed by calculating λ and ϵ values. The λ values calculated according to Eqs. 2 and 3 were, respectively, 0.023 ± 0.007 and 0.022 ± 0.006 μm^{-1}. The corresponding values of ϵ were (13.2 ± 2.8) and (13.8 ± 3.4) × 10^{-17} dyn cm^2.

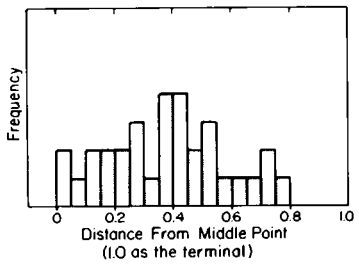

FIGURE 2 Histogram of the distance between the center point of the filament and the point of bending. The half filament length is normalized as 1.

To ascertain whether the decrease in flexibility was due to the effect of calcium ions or to the change in size of the thick filaments following activation, measurements were made on re-relaxed filament suspensions (Kubota et al., 1983). After the activated filaments were re-relaxed by dialyzing against relaxing solution, they did not relengthen (Brann et al., 1979). The elastic modulus of bending as calculated by Eqs. 2 and 3 were, respectively, (12.1 ± 2.5) and (12.6 ± 2.5) × 10^{-17} dyn cm^2, which did not differ significantly from the values for activated filaments. Table I summarizes the λ and ϵ values obtained.

The results show clearly that (*a*) the activated thick filaments tend to be less flexible than the relaxed thick filaments; and (*b*) the activated filaments have practically the same flexibility as that of the re-relaxed filaments. Thus the decreased flexibility of the activated filaments is related to the change in size of the filaments, and not an effect of calcium ions per se, as the shortened, thick filaments are known to be thicker (Dewey et al., 1977; Millman and Hegney, 1983).

The following two facts indicate that the reliability of this method of measuring flexibility is good. Fujime and Kubota (1984) deduced the elastic modulus of bending of relaxed thick filaments of *Limulus* from our light-scattering data (Fan et al., 1983 *a*; Kubota et al., 1984). The ϵ value they obtained was 8.3 × 10^{-17} dyn cm^2. This value agrees nicely with the values reported here. Also, Landau and Liftshitz's equations are derived with minimum free-energy considerations. The only assumption required is that the filaments can be regarded as a homogeneous, continuous entity. Morphologically, the thick filament is not homogeneous in its length. The central bare region lacks cross bridges and is thinner. Nevertheless, the filaments do not show preferential bending at the central bare zone. Fig. 2 illustrates that as far as the bending properties are concerned, the filaments can be regarded as a homogeneous entity.

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The authors are indebted to Dr. S. Fujime for reminding us of the work of Nagashima and Asakura, and to Dr. B. Chu for transmitting the reminder. Also, we wish to thank Dr. S. Goldfine for supplying *Limulus* anti-myosin heavy chain.

Received for publication 5 February 1985 and in final form 15 June 1985.

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