EFFECT OF ATP DEPLETION ON THE ISOLATED THICK FILAMENT OF LIMULUS STRIATED MUSCLE

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ABSTRACT With the dynamic light scattering method, we have shown that calcium ions increase the high-frequency internal motions of isolated thick filaments of *Limulus* striated muscle in the presence of ATP (Kubota, K., B. Chu, Shih-fang Fan, M.M. Dewey, P. Brink, and D. Colflesh. *J. Mol. Biol.* 1983. 166:329 and Fan, S.-f., M.M. Dewey, D. Colflesh, B. Gaylinn, R. Greguski, and B. Chu. *Biophys. J.* 1985. 47:809). If ATP is removed from the suspending medium, an increase of high-frequency internal motions also has been observed with characteristics different from those of filaments suspended in a medium containing ATP and calcium ions. These internal motions appear whether calcium ions are present or not and are suppressed by trifluoperazine (TFP). The motions differ from the calcium ion-induced motions in that (*a*) an energy supply is not required; (*b*) they are insensitive to heat treatment (42°C, 10 min) and (*c*) they are also insensitive to phenylmethylsulfonyl fluoride which blocks the motions in the presence of ATP and calcium ions (Fan, Shih-fang, M.M. Dewey, D. Colflesh, B. Gaylinn, R. Greguski, and B. Chu. 1985. *Biochim. Biophys. Acta.* 827:101). Electron micrographs of negatively stained thick filaments in an ATP-free medium show that the majority of crossbridges extend out from the backbone of the filament and optical diffraction patterns from these filaments lack layer lines arising from the crossbridges. The flexibility of the thick filaments suspended in ATP-free media increases.

When a striated muscle or a contractile system of muscle such as a glycerinated muscle fiber is depleted of ATP, the rigor state develops. The interaction between crossbridges and actin molecules in thin filaments is believed to be the basis for the development of the rigor state. Based on the very sharp fall off in the intensity of the first and second layer lines as they approach the meridian closer than $\sim 1/32^{-1}$ nm in resting frog muscle, Huxley and Brown (1967) argued that in resting muscle the crossbridges extend only out to a radius of 13 nm. This would mean that in a nonactivated muscle at rest length there is a distance of ~6 nm between the end of the crossbridge and the thin filament. The distance is even greater in muscle with shorter sarcomere lengths since the spacing between thick and thin filaments increases as the sarcomere shortens. With quick freezing followed by freeze-etching and rotary metal-shadowing, Ip and Heuser (1983) also showed that in resting rabbit psoas muscle the crossbridges extend out radially only ~15 nm from the backbone of the thick filament. Thus, as muscle goes into the rigor state, crossbridges presumably must first move radially from the backbone of the thick filament before "attaching" to actin filaments. X-ray diffraction studies of frog skeletal muscle in rigor have provided evidence to support this view. H. E. Huxley (1968) and Haselgrove and Huxley (1973) reported that mass moves radially from the thick filament toward the thin filament as the muscle goes into rigor. There are two possible explanations for such movement: (*a*) each crossbridge is affected individually by some change in the sacroplasm when the muscle passes into rigor or (*b*) a change in the backbone of the thick filament allows or causes the crossbridge to move (Haselgrove, 1975).

We have used the quasi-elastic light scattering method to detect calcium-induced high-frequency internal motions in isolated thick filaments of *Limulus* striated muscle suspended in ATP-containing salt solutions (Fan et al., 1983 *a, b,* 1984, 1985 *a, c;* Kubota et al., 1983). If any changes in the internal motions of isolated thick filaments suspended in medium depleted of ATP could be detected, it could give clues to the understanding of the structural changes related to the development of the rigor state.

We have used three methods for studying changes in thick filaments as they go into the rigor state: (*a*) quasi-elastic light scattering studies of isolated thick filaments in suspension, (*b*) optical diffraction of electron micrographs of negatively stained thick filaments, and (*c*) determination of filament flexibility from dark field images of the isolated thick filaments. We have analyzed the data from light scattering studies by comparing the K-dependence of the average line width of the photoelectron count autocorrelation function, $\bar{\Gamma}$, of light scattered in different experimental conditions known to affect cross bridge motions. K is the magnitude of the momentum transfer vector and equals $(4\pi/\lambda)\sin(\theta/2)$, with λ the wavelength of the incident light in the medium and θ the scattering angle. The experimental results obtained show that with ATP depletion (*a*) the $\bar{\Gamma}$ value at $\theta = 120°$ increased about twofold; (*b*) the optical diffraction patterns of negatively

stained thick filaments lack characteristic reflections from myosin heads; and (*c*) the flexibility of the filaments increases. Preliminary results have been reported elsewhere (Chu et al., 1984; Fan et al., 1985*b*).

EXPERIMENTAL METHODS

Preparation of Isolated Thick Filaments

Thick filaments isolated from the levators of the telson of *Limulus* (*Tachypleus polyphemus*) were used. Animals were obtained from Marine Biological Laboratories, Woods Hole, MA, or were caught in Long Island Sound, Belle Terre, NY or the Great South Bay, NY. Muscle bundles were first tied on short wooden sticks at in situ length with the telson in the down position. The bundles were then isolated and soaked in a relaxing solution (100 mM KCl, 5 mM $MgCl_2$, 5 mM Tris, 5 mM EGTA, 2 mM ATP, and 1 mM DTT at pH 7.2 to 7.4) at 4°C overnight. The muscle bundles were then cut into small pieces and homogenized by repeatedly forcing the solution in a syringe through an 18-gauge needle with the tip bent at a nearly 90° angle. After homogenization, the homogenate was first centrifuged at 5×10^3 *g* for 20 min at 4°C. The supernatant was then centrifuged against two-step gradient at 1.2×10^5 *g* for 45 min. The gradient consisted of relaxing solution and relaxing solution mixed with 10 and 60% de-ionized glycerol (vol/vol). The thick filaments were collected at the interface between the 10 and 60% glycerol layer.

ATP was completely depleted by first dialyzing the thick filament suspension with 50 μg/ml hexokinase (Sigma Co., St. Louis, MO; Type V) against a solution that had the identical salt concentration as the relaxing solution, but that contained 2 mM glucose and no ATP at pH 8.0 and was left at room temperature for 2 h (Marston et al., 1976). The pH value of the dialyzing solution was then adjusted to 7.2 ~ 7.4 and the sample was kept at 4°C until the measurement was performed. The filament preparation thus treated is designated hereafter as the "ATP-free sample." The filament suspension dialyzed against an ATP-free solution without the addition of glucose and hexokinase or against a relaxing solution with only very little ATP added, such as <5 μM is called the "ATP-depleted sample." Each thick filament suspension was centrifuged at 5×10^3 *g* for 0.5 to 1 h at 4°C before light-scattering measurement. The purity of the suspension was checked by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis. After depletion of ATP, the ratio of actin/myosin, as determined by means of densitometric tracings of the electrophoretic gels, is <2% of the value of the homogenate. The protein concentration was determined by a modified Lowry method (Fan et al., 1985*c*) and was ~30–100 μg/ml. As the mass of one thick filament of *Limulus* muscle is $\sim 6.4 \times 10^8$ D (Dewey et al., unpublished results), the number of thick filaments in the suspension was $\sim 3 \times 10^{10}$–1×10^{11}/ml. If we take the length, *L*, of the thick filament to be 4.8×10^{-4} cm, then $cL^3 = 3.3$–11, which falls into semidilute regime.

Quasi-elastic Light-Scattering Measurements and Data Processing

The light-scattering spectrometer used and the method of data treatment have been described previously (Kubota et al., 1983; Fan et al., 1985*a*). An argon ion laser operated at 488 nm and ~100 mW was used. Intensity of scattered light was measured by means of an FW 130 photomultiplier tube (ITT Electro Optical Products, Fort Wayne, IN) coupled with an amplifier/discriminator (model 1120; SSR Instruments Co., Princeton, NJ). The emitter-coupled logic pulses were counted for integrated intensity measurements and connected to a digital correlator for Rayleigh linewidth measurement. The average linewidth, $\bar{\Gamma}$, over a large range of *KL* (up to ~100) was calculated. The value of the momentum transfer vector *K* was varied by measuring the scattered light at different scattering angles. *L* is the length of the thick filament.

Electron Microscope Observations

Small droplets of suspensions of filaments were placed on formvar coated copper grids and allowed to rest for 2–3 s. The droplet was then removed by filter paper and the grid surface was flushed with 0.001% Bacitracin (Sigma Chemical Co.) in distilled water. After washing with 0.1 M ammonium acetate, the grids were negatively stained with 2% uranyl acetate. Electron micrographs were obtained at a magnification of 9,800 (microscope magnification calibrated with a grating from Ted Pella Inc., Irvine, CA).

Optical Diffraction of Negatively Stained Isolated Thick Filaments

Optical transforms of images of negatively stained thick filaments were obtained using an Image Analyzer (Rank Industries America, Inc., St. James, MN). The negatives were immersed in optical oil between optical flats. Transforms were recorded on M plates (Kodak Laboratory and Specialty Chemicals, Eastman Kodak Co., Rochester, NY) and spacings were measured using a Joyce-Loebl microdensitometer, MK III.

Measurement of Thick Filament Flexibility

The method used was essentially the same as that reported by Nagahima and Asakuro (1980). The images of thick filaments in a Zeiss Ultrophot with oil immersion dark field condenser (N.A. 1.2/1.4) and planapo 40× oil immersion objective (N.A. 1.0) 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. of America, Long Island City, NY). The contour length of the thick filaments was then measured with a digitizer graphic calculator (model 1224; Numonics Corp., Lansdale, PA) and the end-to-end distance measured directly (Fan et al., 1985*d*). The elastic modulus of bending, ϵ, was calculated with Eq. 1 (Harris and Hearst, 1966).

$$\epsilon = 3k_BT/4\beta, \tag{1}$$

where k_B and T are the Boltzmann constant and the absolute temperature, respectively. The parameter β was calculated with Eq. 2 (Landau and Liftshitz, 1958):

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

where *R* and *L* are the measured end-to-end and the contour length of the thick filament, respectively.

RELEVANT THEORETICAL BACKGROUND FOR INTERPRETATION OF QUASI-ELASTIC LIGHT-SCATTERING RESULTS

There are several possibilities that could increase the $\bar{\Gamma}$ values of filamentous scatterers: (*a*) an increase in the flexibility of the scatterers, (*b*) a decrease in the length of the scatterers, and (*c*) the development of new modes of internal motions. Here we shall describe briefly the relationship among the flexibility of the filaments, the length of the filaments, and the $\bar{\Gamma}$ values.

Relationship between the Flexibility and the $\bar{\Gamma}$ Values

For long flexibile rods, Maeda and Fujime (1981) first developed a theoretical expression for the field correlation function, $g^{(1)}(K, \tau)$. Their expression is based on the Harris

and Hearst model of polymer dynamics (1966) in the dilute regime ($cL^3 \ll 1$, with c being the concentration of the rod in number of rods per milliliter) and in the absence of translation-rotational coupling and hydrodynamic interactions. Recently, Maeda and Fujime (1984) have improved the theoretical model of the field correlation function of light quasi-elastically scattered from solutions of very long rods.

For a semiflexible chain in the dilute regime, each slightly bendable filament is defined by a space curve $\mathbf{r}(s, t)$ having a contour length L and an elastic potential energy, V

$$V = \frac{\xi}{2}\int_{-L/2}^{L/2}\left[\frac{\partial^2 \mathbf{r}(s,t)}{\partial s^2}\right]^2 ds, \quad (3)$$

where ξ is a suitably defined flexural rigidity, s is the coordinate of the line element as measured along the chain, $L/2 = s = -L/2$, and t is the time. By taking into consideration the filament flexibility and anisotropic translational diffusion as well as rotational diffusion, we have

$$\bar{\Gamma}/K^2 \to \left[D - \frac{1}{3}(D_{T\parallel} - D_{T_s})\right] + \frac{L^2}{12}D_R + \frac{k_BT}{\zeta L}\sum_m{}''1, \quad (4)$$

where $D = (2D_{T_s} + D_{T\parallel})/3$ is the average diffusion coefficient, with $D_{T_s} = k_BT/(\zeta L)$ denoting the sideway translational diffusion coefficient, and k_B and T have the usual meaning; $D_{T\parallel} = k_BT/(\zeta_\parallel L)$ denoting the lengthway translational diffusion coefficient; $D_R = 12\,D_{T_s}/L^2$ denoting the end-over-end rotational diffusion coefficient; ζ is the friction constant per unit length of the filament and $\zeta_\parallel = \zeta/2$ in the long rod limit; and $\Sigma_m''1$ denotes the number of bending motions involved in the scattering process.

By taking the long rod limit of diffusion constants and in the absence of hydrodynamic interactions we get $D_{T\parallel} = 2D_{T_s}$, $D = (D_{T\parallel} - D_{T_s})/3 = D_{T_s}$, $(L^2/12)D_R = D_{T_s}$ and $k_BT/\zeta L = D_{T_s}$. Eq. 4 becomes

$$\bar{\Gamma}/K^2 \to 2D_{T_s} + D_{T_s}\sum_m{}''1, \quad (5)$$

which states that each mode of bending motion contributes D_{T_s} to $\bar{\Gamma}/K^2$ when $KL \gg 1$, as in the thick filament case. $\bar{\Gamma}$ values will increase as the flexibility increases.

If the concentration of the filament is in the semidilute regime, then Eq. 4 becomes:

$$\bar{\Gamma}/K^2 \to 2\delta D_{T_s} + D_{T_s}\sum_m{}''1 \quad (6)$$

for $KL \gg 1$ (Maeda and Fujime, 1984). The δ value lies between $1/(cL^3)$ and $1/(cL^3)^2$.

The Relationship between Filament Length and the $\bar{\Gamma}$ Value

The relationship between the length of a rigid rod and the $\bar{\Gamma}$ value is expressed by Eq. 7 (cf. Newmann et al., 1977).

$$\bar{\Gamma} = \frac{k_BT}{3\pi\eta L}\left\{\ln\frac{2L}{d} - \frac{1}{2}\left[1.46 - 7.4\left(\frac{1}{\ln\frac{2L}{d}} - 0.34\right)^2 - 4.2\left(\frac{1}{\ln\frac{2L}{d}} - 0.39\right)^2\right]\right\}K^2. \quad (7)$$

Since the length parameter is in the logarithmic term, the $\bar{\Gamma}$ value is not sensitive to the length change. In Eq. 7, k_B, T, and η are the Boltzmann constant, absolute temperature, and viscosity of the median, respectively, and L and d are the length and the diameter of the rod, respectively.

EXPERIMENTAL RESULTS

Quasi-elastic Light-Scattering Measurements

After the thick filaments were dialyzed against an ATP-free solution with hexokinase and glucose added, the $\bar{\Gamma}$ values at high scattering angle (e.g., 120°) showed a tremendous increase. Fig. 1 shows the results of 16 experiments expressed as the ratio of $\bar{\Gamma}$ values obtained with filament suspended in ATP-free relaxing solution to that suspended in normal ATP-containing solution at a scattering angle of 120°. In each case the relaxed sample and the ATP-free sample were taken from the same muscle preparation. The maximum value of the ratio so far obtained was ~2.6. The mean value of the ratio was 1.97 with a standard deviation of 0.35 (n = 16). Although the $\bar{\Gamma}$ value varied from preparation to preparation, the $\bar{\Gamma}$ value obtained from the same filament preparation under the same experimental condition was remarkably stable. For instance, if we divided a filament preparation into 10 portions, the difference between the $\bar{\Gamma}$ values of each was <3%. If we measured the $\bar{\Gamma}$ values of the same sample five times over a period of 24 h, provided that the sample was kept at 4°C between each measurement, the difference of $\bar{\Gamma}$ values obtained was also <3%. The effect of ATP-free condition was not altered by the presence of calcium ions. β,γ-Imido-adenine-5'-triphosphate (AMP-PNP) prevented the

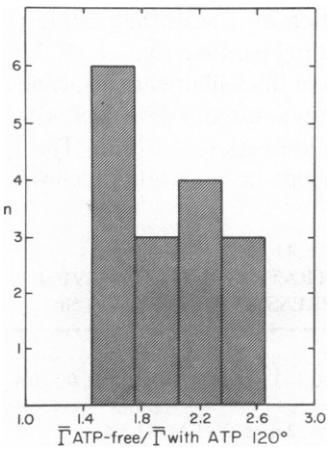

FIGURE 1 Relative increase of $\bar{\Gamma}$ values for *Limulus* thick filaments suspended in ATP-free relaxing solution. The $\bar{\Gamma}$ values obtained with thick filaments suspended in a relaxing solution with ATP were taken as 1.

TABLE I
RELATIVE $\bar{\Gamma}$ VALUES OF THE THICK FILAMENTS SUSPENDED IN VARIOUS ATP-FREE SOLUTIONS (EXPRESSED AS MEAN ± SD)*

	ATP-free relaxing solution‡	ATP-free activating solution§	2 mM AMP-PNP-relaxing solution‖
Relative $\bar{\Gamma}$ value (n = 6)	1.7 ± 0.3	1.8 ± 0.2	1.2 ± 0.1

*Taken at a scattering angle of 90°. The $\bar{\Gamma}$ value of the filaments of the same muscle suspended in regular relaxing solution (i.e., with 2 mM ATP) was taken as 1.
‡100 mM KCl, 5 mM $MgCl_2$, 5 mM Tris, 2 mM EGTA, pH 7.4.
§100 mM KCl, 5 mM $MgCl_2$, 5 mM Tris, 0.1 mM EGTA, 5 mM $CaCl_2$, pH 7.4.
‖100 mM KCl, 5 mM $MgCl_2$, 5 mM Tris, 2 mM EGTA, 2 mM AMP-PNP, pH 7.4.

increase of $\bar{\Gamma}$ values by ATP-free treatment. The possible ATP contamination in AMP-PNP was controlled by prior treatment with hexokinase and glucose. Table I gives the $\bar{\Gamma}$ values from thick filaments suspended in the ATP-free relaxing solution, in ATP-free activating solution (relaxing solution with 5 mM $CaCl_2$ added and the concentration of EGTA lowered to 0.1 mM), and in ATP-free relaxing solution with 2 mM AMP-PNP added. The $\bar{\Gamma}$ values are expressed in such a way that the $\bar{\Gamma}$ value of the same filament preparation suspended in relaxing solution is taken as 1. As previously demonstrated with ATP-containing relaxing solution (Fan et al., 1985*a*), the addition of calcium ions to the relaxing solution in which AMP-PNP was substituted for ATP did not affect the $\bar{\Gamma}$ values. Magnesium ion concentrations between 1 and 5 mM had no effect on the $\bar{\Gamma}$ values from filaments suspended in ATP-free solution (Table II). As the pH value of the suspending medium was lowered to ~6, the addition of ATP to the ATP-free medium reduced the $\bar{\Gamma}$ values to that obtained from the ATP-containing relaxing solution. pH 6 is close to the isoelectric point of the thick filaments as determined with Donnan potential measurements (Dewey et al., 1982). If the pH value of the medium remained at 7.2–7.4, the addition of ATP did not lower the heightened $\bar{\Gamma}$ value (Table III). The $\bar{\Gamma}$ value of the thick filaments suspended in ATP-free solution at a scattering angle of 120° is usually ~10% lower at pH 6.0 than that at pH 7.0.

The increase of $\bar{\Gamma}$ values from thick filaments suspended in ATP-depleted relaxing solution usually developed after the ATP concentration was lowered to <5 μM. The $\bar{\Gamma}$ values of filaments suspended in activating solution

TABLE II
EFFECT OF Mg^{2+} CONCENTRATION ON RELATIVE $\bar{\Gamma}$ VALUES AT θ = 120° (EXPRESSED AS MEAN ± SD)

Mg^{2+} concentration (mM)*	1	2	3	5
Relative $\bar{\Gamma}$ values (n = 6)	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.2	1.9 ± 0.1

*Containing 100 mM KCl, 5 mM Tris, 2 mM EGTA, pH 7.4.

TABLE III
EFFECT OF pH ON THE REVERSIBILITY OF THE RELATIVE INCREASE OF $\bar{\Gamma}$ VALUES DUE TO ATP-FREE CONDITION (MEASURED AT θ = 120°, $\bar{\Gamma}$ VALUES EXPRESSED AS MEAN ± SD)

pH	6.0	7.2
In ATP-free medium*	1.8 ± 0.2	2.1 ± 0.2
After the addition of 2 mM ATP‡	1.0 ± 0.1	2.1 ± 0.2
Re-dialyzed against ATP-free medium*	1.7 ± 0.2 (n = 6)	2.1 ± 0.2 (n = 8)

*100 mM KCl, 5 mM $MgCl_2$, 5 mM Tris, 2 mM EGTA, pH 7.4.
‡100 mM KCl, 5 mM $MgCl_2$, 5 mM Tris, 2 mM EGTA, 2 mM ATP, pH 7.4.

decreased with the ATP concentration as the ATP concentration was lowered from 2 mM to ~10 μM and then increased as the ATP concentration was lowered to 5 μM and less (Fig. 2).

Although both calcium ions and an ATP-free condition increased the $\bar{\Gamma}$ values of thick filament suspensions, the effect of the latter was different from that of the former (Fan et al., 1983*a*; Kubota et al., 1983; Fan et al., 1985*a*) in the following ways. (*a*) The increase of $\bar{\Gamma}$ values in the ATP-free condition did not require energy. Energy from the hydrolysis of ATP is indispensable for the increase of $\bar{\Gamma}$ values induced by calcium ions (Fan et al., 1985*a*). (*b*) The increase of $\bar{\Gamma}$ values in ATP-free solution was not sensitive to heat treatment (42°C, 10 min). (*c*) The increase of $\bar{\Gamma}$ values in ATP-free solution was insensitive to phenylmethylsulfonyl fluoride (PMSF). The increase of $\bar{\Gamma}$ values resulting from calcium ions is sensitive both to heat treatment (Fan et al., 1985*a*) and to PMSF (Fan et al., 1985*c*). The results are presented in Table IV. The $\bar{\Gamma}$ values are expressed in the same way as in Table I. We have shown that the effect of PMSF is dependent on the concentration ratio of PMSF to protein (Fan et al., 1985*c*). The protein concentration used for testing the effect of

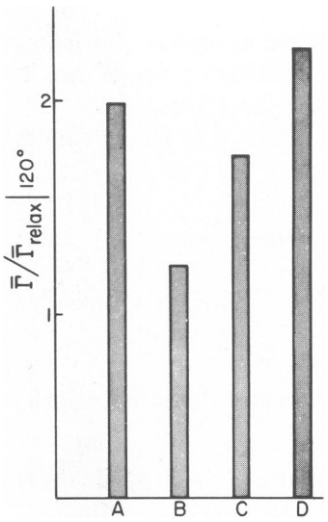

FIGURE 2 Relationship between the ATP concentration in the activating solution and the $\bar{\Gamma}$ values of the suspended filaments. *A*, *B*, *C*, and *D* are the results obtained, respectively, in solutions containing 2 mM, 10 μM, 3 μM, and 1 μM ATP.

TABLE IV
EFFECTS OF HEAT TREATMENT AND PMSF ON RELATIVE $\bar{\Gamma}$ VALUES AT θ = 120° (EXPRESSED AS MEAN ± SD)

	Activating solution*		ATP-free activating solution‡	
Control	2.1 ± 0.1	2.4 ± 0.3	2.2 ± 0.2	2.1 ± 0.2
After 42°C, 10 min	1.0 ± 0.1		2.2 ± 0.2	
After 1 mM PMSF		1.2 ± 0.1		2.1 ± 0.2
	(n = 10)	(n = 7)	(n = 6)	(n = 6)

*100 mM KCl, 5 mM $MgCl_2$, 5 mM Tris, 0.1 mM EGTA, 5 mM $CaCl_2$, 2 mM ATP, pH
‡100 mM KCl, 5 mM $MgCl_2$, 5 mM Tris, 0.1 mM EGTA, 5 mM $CaCl_2$, pH 7.4.

PMSF on the increase of $\bar{\Gamma}$ values induced by ATP-free condition was ~30 μg/ml. At such protein concentrations, 1 mM PMSF suppresses, almost completely, the increase of the $\bar{\Gamma}$ values due to calcium ions.

We have shown that pretreatment with trifluoperazine (TFP) suppresses the increase of $\bar{\Gamma}$ values caused by calcium ions (Chu et al., 1984). TFP also suppresses the increase of $\bar{\Gamma}$ values in ATP-free solutions. Table V gives the results.

Electron Micrographs of Negatively Stained Isolated Thick Filaments and Their Optical Transforms

Electron micrographs of isolated negatively stained thick filaments from *Limulus* treated with an ATP-free solution showed crossbridges extending out from the backbone. These images, when optically transformed, lacked the characteristic reflections at 14.6 nm (Fig. 3 *B*). Optical transforms of images of negatively stained isolated thick filament in relaxing solution clearly demonstrate the characteristic reflections (Fig. 3 *A*).

Measurements of Thick Filament Flexibility

The contour length and the end-to-end length of the relaxed thick filaments measured were 4.84 ± 0.25 μm and 4.56 ± 0.38 μm, respectively (mean ± SD, n = 38). The elastic modulus of bending calculated was (9.2 ± 1.9) × 10^{-17} dyn · cm (mean ± SD, Fan et al., 1985*d*). After dialyzing against ATP-free solution, the corresponding lengths were 4.66 ± 0.18 μm and 4.28 ± 0.37 μm (mean ± SD, n = 29), respectively. The elastic modulus of bending calculated from Eqs. 1 and 2 was (7.2 ± 1.2) × 10^{-17} dyn · cm (mean ± SD).

TABLE V
EFFECTS OF TFP ON RELATIVE $\bar{\Gamma}$ VALUES AT θ = 120° (EXPRESSED AS MEAN ± SD)

	Activating solution*	ATP-free activating solution‡
Control	2.0 ± 0.2	2.0 ± 0.2
After 50 μM TFP	1.1 ± 0.1	1.1 ± 0.2
	(n = 7)	(n = 6)

*The composition is the same as that given in the footnote * of Table IV.
‡The composition is the same as that given in the footnote ‡ of Table IV.

DISCUSSION

Dynamic light-scattering studies of the isolated thick filaments of *Limulus* muscle reveal an increase in the average linewidth, $\bar{\Gamma}$, of the autocorrelation photocount function as ATP is removed from the suspending medium. Images of the filaments in the dark field microscope show that the average length of the filaments is slightly decreased but that the flexibility calculated is increased under identical conditions. These changes in parameters of the filaments have the following effects on the $\bar{\Gamma}$ values. For rod-like structures the relationship between length and $\bar{\Gamma}$ values is described in Eq. 7. When the length of a rod is decreased from 4.84 to 4.66 μm the $\bar{\Gamma}$ value increases <5%, assuming that the initial diameter of the rod is ~25 nm and increases during filament shortening to maintain constancy of filament density. Such an increase (<5%) is practically insignificant as the $\bar{\Gamma}$ values of the thick filaments suspended in ATP-free solution increase 97% on average.

An increase in the flexibility of the rods alone cannot account for the increase in the $\bar{\Gamma}$ values observed. The average $\bar{\Gamma}/K^2$ value at θ = 120° of thick filaments suspended in relaxing solution with ATP is ~$3D_{Ts}$. The $\bar{\Gamma}/K^2$ value increased to ~$6D_{Ts}$ on average and $8D_{Ts}$ at a maximum for thick filaments suspended in ATP-free solution. For the concentration of thick filaments that we used, the value of $\bar{\Gamma}/K^2$ should not exceed 3–$4D_{Ts}$, if no other internal motion in the rods developed. Since $\bar{\Gamma}/K^2$ increased to a value that exceeded this, the increase must have resulted not only from the increase in flexibility of the filaments but also from the development of additional internal motion(s) in the filament (Fan et al., 1987).

Another method of evaluating whether the increase of $\bar{\Gamma}$ values can be explained solely by the increase in flexibility of the filaments is to examine the relationship between $\bar{\Gamma}/K^2$ vs. K^2 from the data obtained with samples suspended in ATP-free medium as shown in Fig. 4. The $\bar{\Gamma}$ values we have used are from the sample with minimal increases. By using the relation between the elastic modulus of bending, ϵ, and the persistence length, γ, $\gamma = k_BT/2\epsilon$, we obtain $\gamma = 2.86 \times 10^2$ cm^{-1} for $\epsilon = 7.2 \times 10^{-17}$ dyn · cm and $\gamma L = 0.133$ for $L = 4.66$ μm. The theoretical curve $\bar{\Gamma}/K^2$ vs. K^2 for $\gamma L = 0.133$ (cf. Maeda and Fujime, 1984) is shown as a dotted line in Fig. 4. Fig. 4 shows that even in the particular experiment that the increase of $\bar{\Gamma}$ values after ATP-depletion is relatively small, the increase cannot be accounted for entirely by an increase in the flexibility of the filaments. If it were due only to an

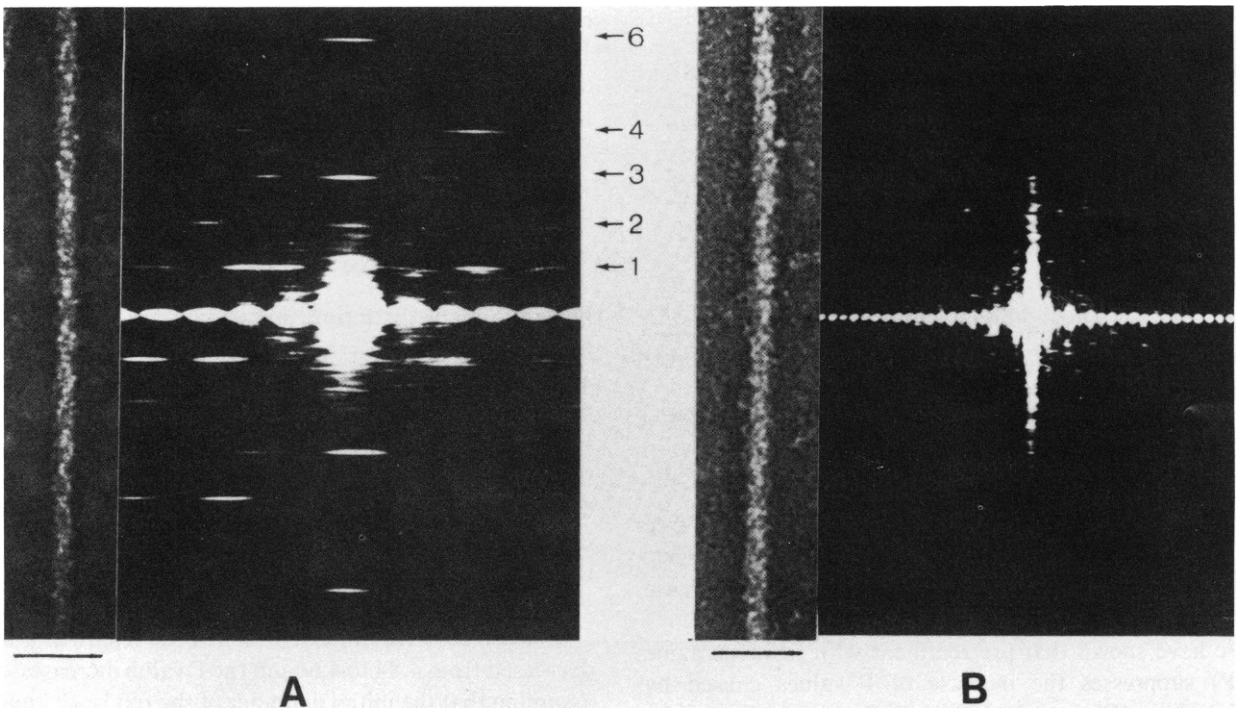

FIGURE 3 Electron micrographs of thick filaments suspended in regular relaxing solution (*A*) and ATP-free relaxing solution (*B*) and their optical transforms. The relaxing solution contains 100 mM KCl, 5 mM $MgCl_2$, 5 mM Tris, 2 mM EGTA, 2 mM ATP, pH 7.4. Horizontal bar equals to 100 nm. For further explanations, see text.

increase in the flexibility of the filaments then γL should increase to ~0.5. This would require that the elastic modulus of bending be as low as 1.9×10^{-17} dyn · cm. This value is incompatible with the appearance of the images of the filaments in suspension observed with the dark-field microscope. Thus, again it seems that we should look for the development of additional modes of motion as an explanation for the increased values of $\bar{\Gamma}$. Considering the structure of the thick filament, the most plausible new mode of motion to develop might be the movement of the crossbridges. Electron micrographs of the isolated filaments are consistent with this interpretation since they reveal that the crossbridges extend from the filament backbone under these conditions. The crossbridge positions appear random in such treated filaments, as if the crossbridges are moving randomly due to thermal agitation. Recently, Levine et al. (1986), using negatively stained isolated thick filaments, reported similar crossbridge radial shifts in ATP-free conditions. They also reported that the effect was reversible, but they did not give the pH values of the solutions they used. The increase of the flexibility of the isolated thick filaments is also expected if the crossbridges detach from the filament backbone.

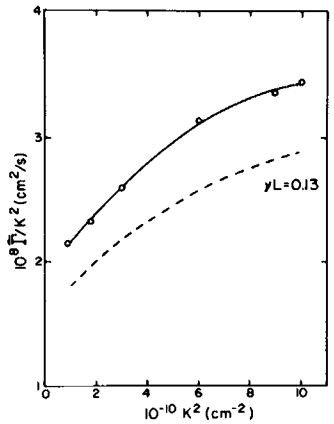

FIGURE 4 Plot of $\bar{\Gamma}/K^2$ versus K^2 for thick filaments suspended in ATP-free solution (*solid line*, data from the experiment with minimal increase of $\bar{\Gamma}$ values) and the theoretical curve of a suspension of long filaments ($L = 4.66$ μm) with $\gamma L = 0.13$.

The increase of $\bar{\Gamma}$ value due to ATP-depletion develops as the ATP concentration is lowered below 5 μM. This recalls the observations of Maruyama and Weber, 1972 (see also Bremel and Weber, 1972). They reported that when, in vertebrate myofibrils, the ATP concentration was lower than 8 μM, the rigor state develops. Since the crossbridges should first swing out before they attach to the thin filament as the rigor state develops, their results also suggest that as the ATP concentration is lowered to less than several micromolars, the crossbridges move radially.

As discussed in the Introduction, for a muscle at rest, there is a short fall of several nanometers between the end of the crossbridge and the thin filament when the muscle is at rest. The short fall is even greater in muscle with shorter sarcomere lengths. In the case of rigor, the crossbridges must first move radially towards the thin filament before they interact with the actin molecules in the thin filament. The radial movement of the crossbridge must be an indispensible step in the development of the rigor state. It is not clear whether the mechanism of radial movement of

crossbridges of isolated thick filaments in an ATP-free condition is similar to that which happens during rigor. Nevertheless, any understanding about the mechanism(s) involved in inducing the radial movement might serve as a clue to deciphering the events occurring during the establishment of rigor in muscle.

Under our experimental conditions, the ATP depletion effect is reversible by the addition of ATP only when the pH value of the medium is close to the isoelectric point of the filament matrix. This seems to indicate that a crossbridge can attach to the filament backbone only when it can move close to the backbone during the random motion due to thermal agitation. Electrostatic repulsion due to surface charges at pH values away from the isoelectric point might prevent it from doing so.

The treatment of the contractile apparatus with ATP-free solutions similar to those we have used in this work has been used extensively in the study of crossbridge mechanisms (for instance, Thomas and Cooke, 1980 and Cooke et al., 1982 in their electron paramagnetic resonance studies; Yanagida, 1981 in his polarized fluorescence studies; Burghardt et al., 1983 in their dichroic studies). Radial shift of the crossbridge should occur before the establishment of the rigor state. Further understanding of the radial movement of the crossbridge in ATP-free condition would be helpful in the analysis of experimental results involving the use of the ATP-free condition.

Wray (1984) reported that AMP-PNP does not produce any observable change in the x-ray diffraction pattern from *Limulus* muscle but does cause a small (10%) loss in tension. He suggested, based on the observations of *Limulus, Lethocerus,* and crayfish muscles, that the effect of AMP-PNP is mainly on the crossbridge itself and not on the attachment of the myosin head to the actin filament. The x-ray diffraction studies of Padron and Huxley (1984) on frog sartorious muscles treated with AMP-PNP showed that the largest effect induced by AMP-PNP on the diffraction pattern of rigor muscle was in the myosin reflection. This was interpreted as an indication that the structure of the crossbridge close to S-2 changed significantly. In view of these suggestions and our finding that AMP-PNP can substitute for ATP in keeping the crossbridges from detaching from the filament backbone, it would seem that either (*a*) the radial movement of the crossbridge is affected by ATP at a different site than the myosin ATPase site, which is believed to be involved in the detachment of the crossbridge from actin during cycling, or (*b*) that AMP-PNP and ATP act on different sites that affect crossbridge attachment. The fact that myosin has more than one binding site for ATP has been suggested by the work of Levy and Ryan (1967) and Pemrick and Martinez (1985).

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