Proc. Natl. Acad. Sci. USA
Vol. 76, No. 12, pp. 6346–6350, December 1979
Biophysics

Fluctuations in polarized fluorescence: Evidence that muscle cross bridges rotate repetitively during contraction

(fluorescence correlation spectroscopy/muscle contraction)

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ABSTRACT **Particular thiols of the myosin subfragment 1 moieties of single glycerinated muscle fibers are covalently labeled with rhodamine. By using appropriate solutions such fibers can be relaxed, be in rigor, or develop active isometric tension. The rhodamine is excited by polarized 514.5-nm laser light; the >580-nm fluorescence is resolved into orthogonal components and the intensity of each is measured by a computer-interfaced photon counting system. Fluctuations over-and-above noise appear in steady-state activity but not in relaxation or rigor and not when the fluorophore is actin-attached instead of myosin-attached. Fluctuations also appear in *ratios* of polarized intensities—quantities sensitive to fluorophore attitude but not to fluorophore number. The fluctuations are dominated by low (≈2 Hz) frequencies similar to separately measured ATPase frequencies. The fluctuations are ascribed to repetitive motion of the cross bridges to which the rhodamine is attached.**

The "cycling cross bridge" theory of muscle contraction postulates that myosin projections ("S-1" moieties) execute repetitive rotational motion coupled (1) to repetitive (enzymatic) chemistry occurring at myosin S-1 ATPase sites. According to this theory, a cross bridge attaches to actin at a specific attitude (2, 3)—e.g., 90° ("relaxation") to the fiber axis. Attachment is followed by a "power stroke" in which the S-1 "rolls" on actin (2), passing through many states down to, say, 45° ("rigor"). After the roll and the binding of a new ATP, the moiety detaches from actin. Detachment allows reorientation to 90°. A new cycle begins on the next attachment; if shortening occurs, attachment is to a "downstream" actin site. This theory is supported by much circumstantial evidence but not yet by direct test. One aspect—on stimulation is there a *transient* increase in myosin-actin attachments?—is being investigated elsewhere by time-resolved x-ray diffraction (4, 5). Here we attempt to test directly whether in steady-state (isometric) tension development the cross bridges execute repetitive rotational motion.

The ideas underlying our examination of the theory follow. The steady-state tension (or the time-averaged value of any other cross bridge-related parameter) may, as the theory assumes, be the summation of the individual activities of a large population of molecular impellers behaving stochastically. Therefore, if the parameter is measured with sufficient sensitivity there will be revealed fluctuations around the "average" or steady-state value of the parameter. A Fourier analysis of these fluctuations gives an account of the frequencies that compose them. Of course, we must be sure that the fluctuations are "over-and-above" those generated by measuring instruments or other artifactual sources. What we will report (interchangeably) about such analyzed fluctuations is their "autocorrelation function", $G(\tau)$, and their "power spectrum", $S(\nu)$. $G(\tau)$ expresses the similarity between two measurements of the parameter separated in time by τ; in particular, the "half-time" [at which $G = G(0)/2$] of $G(\tau)$ is a simple characteristic of the rapidity of fluctuations. $S(\nu)$ is a plot against frequency, ν, of the square of the Fourier coefficient for that frequency; thus $S(\nu)$ tells "how important" frequency ν is in composing the observed fluctuations. The theory assumes that the cross bridges are in motion while MgATP is being hydrolyzed by the fiber and tension is being developed, but it also assumes that they are motionless when either (*a*) contact with actin is prevented by the regulatory proteins ("relaxation" state) or (*b*) MgATP is absent and the cross bridges are "permanently" attached to actin ("rigor" state). Therefore, it predicts that $G(\tau)$ and $S(\nu)$ will be different during activity than during relaxation or rigor and that any fluctuations observed in the static states are instrumental or other artifactual "noise"; such noise would also be included in "active" fluctuations. Turning the reasoning around, if there prove to be significant differences in the active state compared to the static states we will conclude that going on during activity is a motion composed of the frequencies newly appearing during activity. We shall consider that the motion is *repetitive* if for different time intervals the fluctuations generate the same $G(\tau)$ and $S(\nu)$. $G(\tau)$ or $S(\nu)$ can be related to specific chemical schemes or "models" of contraction (see ref. 6) by stochastic simulation on a computer, observation of the computer-generated $S(\nu)$, and, finally, comparison with the experimentally observed $S(\nu)$ (7, 8). Here however, the sole question is whether there is repetitive cross-bridge motion during the active state.

Previously we recorded and analyzed steady state fluctuations in fiber tension (7) and in polarized intensities of fluorescence originating in dyes uniquely attached to cross bridges. In both cases $S(\nu)$ during tension was dominated by frequencies of 1–5 Hz, whereas in relaxation or rigor $S(\nu)$ was flat. Also, we found the ATPase frequency to be in this range (9). But in the tension measurements we could not be sure that the motion was *originating* in the cross bridges, and in the intensity measurements we could not exclude that some cross bridge-unrelated pulsation might be merely towing cross bridges (therefore fluorophores) in and out of the field of view.

In this work both hazards are overcome. The fluorescent labels are for the most part attached to the "SH_1" thiols of the cross bridges, and we measure *ratios* of polarized fluorescence intensities from which fluorophore *number* cancels out: $R_\perp \equiv I_{\perp\perp}/I_{\perp\parallel}$, and $P_\perp \equiv (I_{\perp\perp} - I_{\perp\parallel})/(I_{\perp\perp} + I_{\perp\parallel})$.* Our previous work (10–14) has shown that ratios of the I_{ij}s* can be re-

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Abbreviations: $G(\tau)$, autocorrelation function; $S(\nu)$, power spectrum; IATR, iodoacetamidotetramethylrhodamine; IAF, iodoacetylfluoresceine; IAEDANS, (*N*-iodoacetyl-*N'*-1-sulfo-5-naphthyl)ethylenediamine.

* I_{ij} means that the plane of excitation is in relation i to the fiber axis and that the plane of observation of the emission is in relation j to the fiber axis.

lated to the attitudinal angles of the S-1 moieties. The demembranated single fibers used are always in equilibrium with 80 mM KCl/5 mM $MgCl_2$/5 mM phosphate, pH 7.0, at room temperature; for "rigor" 2 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) was also included; for "relaxation" 2 mM EGTA and 5 mM ATP were included; for "tension development" 0.1 mM $CaCl_2$ and 5 mM ATP were included.

METHODS

The hardware for detection of polarized fluorescence fluctuations is depicted in Fig. 1.

Analysis of photon counting data stored in the computer disc is as follows. In each bin the photon counts are divided by the monitor counts and then by each other to give R; then their difference is divided by their sum to give P. The four sequences of numbers are partitioned into "sweeps" of 1024 numbers each. The data in each sweep are fitted (least squares) to a straight line. Each point on the line is subtracted from the corresponding experimental point to correct for the slow drift in fluorescence intensity due to photobleaching. The resulting sequence of 1024 points is treated as described (7). The procedure is repeated on subsequent sequences (usually 120) and the resulting power spectra are averaged. The spectra always show 40-Hz and 80-Hz peaks, due to aliasing of laser intensity fluctuations at 360 Hz, that are not completely compensated by the monitoring system. The peaks are digitally filtered out; the resulting $S(\nu)$s are inverse Fourier transformed to give the $G(\tau)$s.

The signal-to-noise (S/N) ratio in our apparatus is mainly determined by the number (m) of fluorescent photons detected during one bin width per fluorophore (labeled cross bridge) (16, 17). m is proportional to the collection efficiency (g), extinction coefficient of fluorophore (ϵ), its quantum yield (Q), the bin width (Δt), and the laser light flux ($F = P/\pi\omega^2$). In a typical iodoacetamidotetramethylrhodamine (IATR) experiment, $g = 0.007$, $\epsilon = 3 \times 10^4\ M^{-1}\ cm^{-1}$, $Q = 0.9$, $\Delta t = 5$ ms, $P = 4.8 \times 10^{12}$ photons s^{-1}, and $\omega = 7\ \mu$m; then m is 2.2×10^{-2}. S/N also depends on the total number of bins counted (16), and the half-time for decay of $G(\tau)$; typically, these numbers were 81,920 and 500 msec, respectively. Using the foregoing, S/N = 5.8. Such S/N ratios are attainable only because we are observing inherently slow rotations.

The depth of focus of the objective was *ca.* 20 μm, and the illuminated volume was *ca.* 500 μm^3; we calculate that approximately $1/10$th of the S-1 moieties in the fiber are labeled with the fluorophore. Thus the total number of fluorophores in the illuminated volume is estimated at 2.3×10^6. This number is consistent with the observed rate of photon arrival at the photomultipliers.

To prepare specimens, rabbit psoas muscle was cut into thin strips, tied to plastic sticks at body length, and immersed in 138 mM KCl/2 mM MgATP/5 mM EGTA/10 mM imidazole/6 M glycerol at pH 7.0; it was stored in this solution for 1–30 weeks at −18°C. Fibers are never in rigor prior to use (18); they can sustain steady isometric tension for at least 15 min and seldom break. Single fibers were dissected out, skinned, and mounted as described (7). Myosin S-1 in fibers was labeled with 0.155 mM iodoacetyl label (usually IATR); actin labeling was with iodoacetylfluoresceine (IAF) in rigor solution for 30 min at room temperature in the dark without stirring. Labeled fibers could be stored for up to 48 hr in the dark. They were thoroughly washed with relaxation solution prior to use.

Specificity of labeling was assessed by electrophoresis in polyacrylamide gels. Fibers were dissolved in 1% sodium dodecyl sulfate/6 M urea/1% 2-mercaptoethanol/10 mM $NaPO_4$/10 mM EGTA at pH 7. The gel tubes were photographed immediately after electrophoresis, and photonegatives were used for densitometry.

Measurement of ATPase activity of isometrically held single fibers is described elsewhere (9).

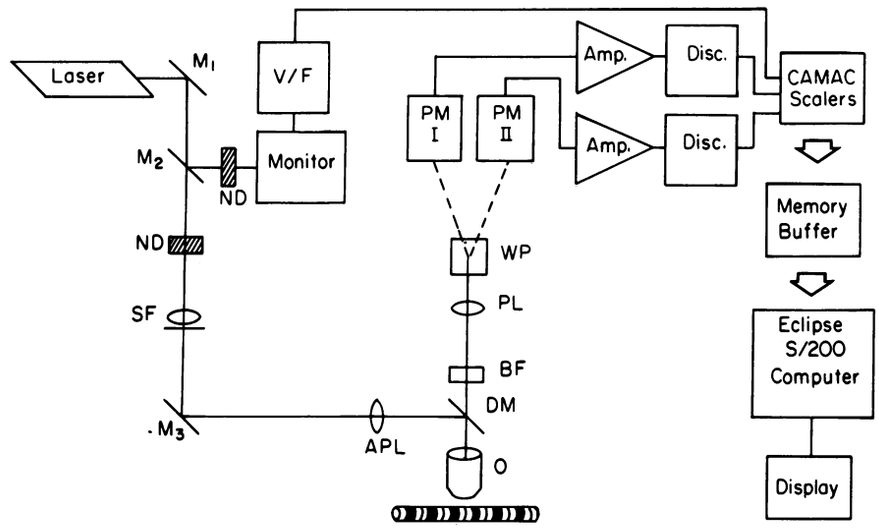

FIG. 1. Fluorescence correlation spectrometer (15). An argon laser generates a vertically polarized 514.5-nm beam of Gaussian profile. The beam is attenuated by neutral density filters (ND) and directed by mirrors (M) to the microscope objective (O) that both focuses excitation on the sample and collects the fluorescence. A spatial filter (SF) improves the beam profile and an auxiliary positioning lens (APL) adjusts the focus of the beam on the focal plane of the objective. A dichroic mirror (DM) is a beam splitter reflecting all wavelengths less than 580 nm. A barrier filter (BF) further blocks exciting light, and a projector lens (PL) projects the fluorescence onto a Wollaston prism (WP) which splits it into two orthogonally polarized beams diverging by 20°. Each beam strikes a photomultiplier, followed by an amplifier and a discriminator arranged so that a single pulse results from a single photon. A small fraction of the beam is directed to the monitor photodiode, followed by an amplifier and a voltage-to-frequency converter; this tap-off compensates for laser intensity variations. Photomultiplier and monitor pulses are counted by a CAMAC Quad Scaler over a period of time (the "bin width"). A series of 16-bit integer numbers representing photomultiplier and monitor counts accumulated in a bin interval is stored on files on a computer disc for subsequent analysis. The optical apparatus rests on a vibrationless table. The light beams are in tubes to minimize intensity fluctuations due to dust.

EXPERIMENTAL PITFALLS

In these experiments there are points at which error or misconception can arise. Some of these pitfalls have been anticipated, and hopefully avoided, by this or by previous work.

Fluorescence Labeling. When we are presuming to detect myosin S-1 motion it is essential that the fluorescence actually originates in S-1 moieties. By using other iodoacetamide derivatives, we have shown that when a glycerinated fiber is labeled in the relaxed state the "SH_1" thiols of the S-1 moieties are labeled specifically (14). Fig. 2 shows, as with (*N*-iodoacetyl-*N'*-1-sulfo-5-naphthyl)ethylenediamine (IAEDANS) and IAF, that myosin heavy chain (site of SH_1) is also predominantly labeled with IATR. Furthermore, the Ca^{2+}-ATPase of myosin labeled with IATR was activated 5-fold, as would be expected from reacted SH_1.

Previously (14), and in this work, the Ca^{2+} response and the tetanic tension of fibers was not appreciably affected by labeling. Also, there are arguments for thinking that dye bound to S-1 is sufficiently steady so that in the time scale of interest S-1 motion can be inferred from attached label motion (19); if this were not so our motion results during tension development would resemble those in the static states.

Light Scattering and Birefringence. By using nonabsorbed wavelengths we have shown with progressive improvements that light scattered is less than 3% (10) or 1% (14) of light fluoresced. Here it was less than 0.1%.

As is light scattering, birefringence is a property of the regions through which excitation and emission pass. During isometric steady-state activity these properties assume some time-average values; fluctuations away from these averages are much less than the differences between static states—i.e., *all* cross bridges in relaxation and then all in rigor. Differences in total birefringence, Δn, have been reported for intact fibers under certain conditions (20); therefore, we examined our specimens for differences in Δn between extrema, assuming that fluctuations would be smaller than such differences. The retardation, Φ (cm), was measured by using a quarter-wave plate and 546.1-nm radiation; fiber thickness, d (cm), was measured by calibrated top-and-bottom focusing. The relaxation-to-rigor transition was made in steps—ATP relaxation to pyrophosphate (10 mM PP_i/80 mM KCl/2 mM $MgCl_2$/1 mM EGTA at pH 7) relaxation to rigor—in order to avoid contractions (thus d changes) during the disequilibrium of solution exchange. In 20 fibers we found no difference between retardation extrema ($P = 0.6$) and $\Phi_{average} \pm SEM = 54 \pm 1.7$ nm. Extreme differences in $\Delta_n = \Phi/d$ were also nil, but variance was much larger due to difficulty in measuring d. Because extreme differences in Δn were nil we concluded that, *a fortiori*, fluctuations in birefringence contribute nothing to fluctuations in R or P.

Another observation reassured us. When fluorophores were on the actin rather than on the S-1 component, fluorescence fluctuations were greatly reduced or absent, even though the excitation and emission were still passing through the same fiber regions in which Δn might have been fluctuating (see Fig. 5*B*).

Photobleaching. None of the fluorescent labels thus far tried (IAEDANS, IAF, and IATR) was completely stable under laser illumination. During the experiment the photodegradation caused a drift in total fluorescence intensity (Fig. 3*A*); if uncompensated the drift would produce a slow decay in $G(\tau)$. However, the foregoing data processing compensated for the

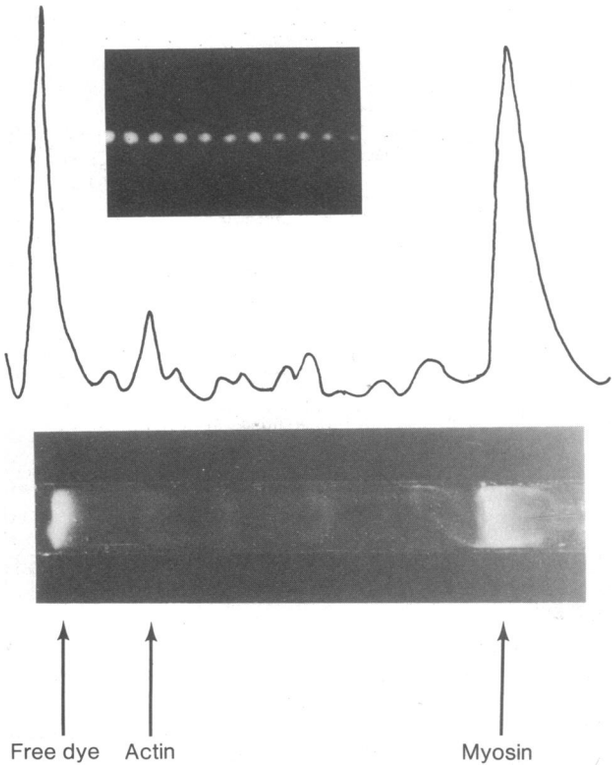

FIG. 2. Electrophoretogram of a fiber stained with fluorescent IATR. Because gels are unfixed (to avoid quenching of IATR fluorescence), photographs were taken immediately on completing the run. (*Inset*) Fluorescence photomicrograph of IATR-labeled myofibril showing predominant A-band labeling.

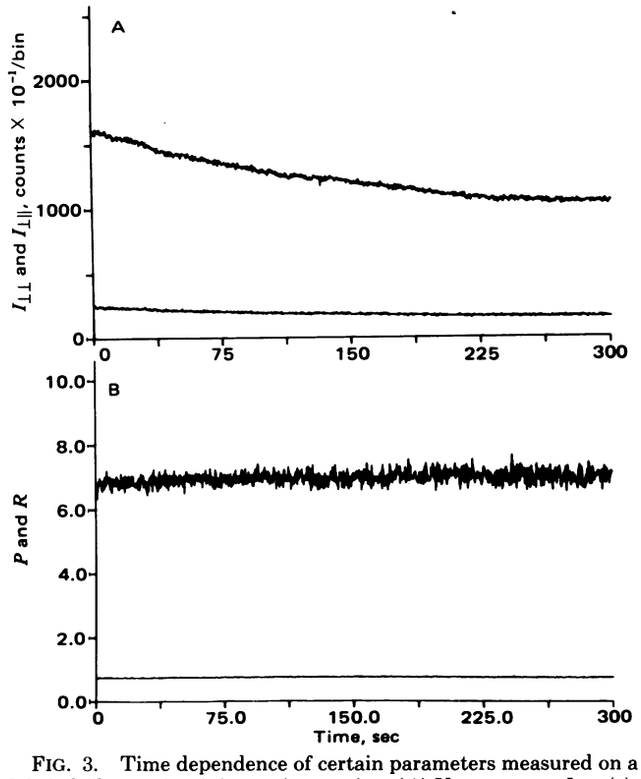

FIG. 3. Time dependence of certain parameters measured on a labeled fiber maintaining active tension. (*A*) Upper curve, $I_{\perp\perp}(t)$; lower curve, $I_{\perp\parallel}(t)$. (*B*) Upper curve, $R_\perp(t)$; lower curve, $P_\perp(t)$. For the $I(t)$, 15,935 ± 121 ⊥ photons per bin and 2353 ± 47 ∥ photons per bin were counted over the first 1 sec. After 150 sec these numbers had dropped, due to photodegradation, to 11,883 ± 120 and 1702 ± 42, respectively. $R_\perp(t)$ was 6.772 ± 0.14 over the first 1 sec and 6.984 ± 0.18 after 150 sec. $P_\perp$ was 0.742 ± 0.0046 over the first 1 sec and 0.749 ± 0.0057 after 150 sec.

drift adequately, as shown by the flatness of $G(\tau)$ despite the photobleaching (Fig. 4*A*).

Number Fluctuations. It is conceivable that during sustained activity an6elastic fiber suffers a gross pulsation (e.g., a contracting sarcomere stretching its neighbors) that moves fluorophores in and out of view. If the fluorophores are not densely distributed throughout the fiber any I_{ij} will exhibit fluctuations due to this pulsation. Four types of results lead us to think that such a pulsation is either absent or at least does not mask the attitudinal fluctuations of interest: (*a*) The fluctuations in I_{ij} produced by *towing* the fiber across the field of view are such that in order to mimic the frequencies we observe we calculate (21) that the towing rate would have to exceed the free shortening velocity of contraction. If such a motion actually occurred, it would be easily visible under the microscope, and it is not. (*b*) As we have stressed earlier, in theory any observed I_{ij} is proportional to fluorophore number (N), but a *ratio* of two I_{ij}s (R,P) is independent of N and should therefore be insensitive to fluctuations in N. (*c*) The rate of photobleaching is about the same in tension development as it is in relaxation and rigor. If there existed a gross pulsation in active muscle, even as slow as 10 μm min^{-1}, nearly all fluorophores in the illuminated volume would be replaced within 60 sec. Such replacement would make photobleaching slower during activity, which is not observed (Fig. 5*A*). (*d*) If what we observe in fluorescence were N-fluctuations due to a gross pulsation, then we might continue to observe such fluctuations no matter to what component the fluorescent labels are attached. But when the labels are on the actin filaments the fluctuations disappeared (Fig. 5*B*).

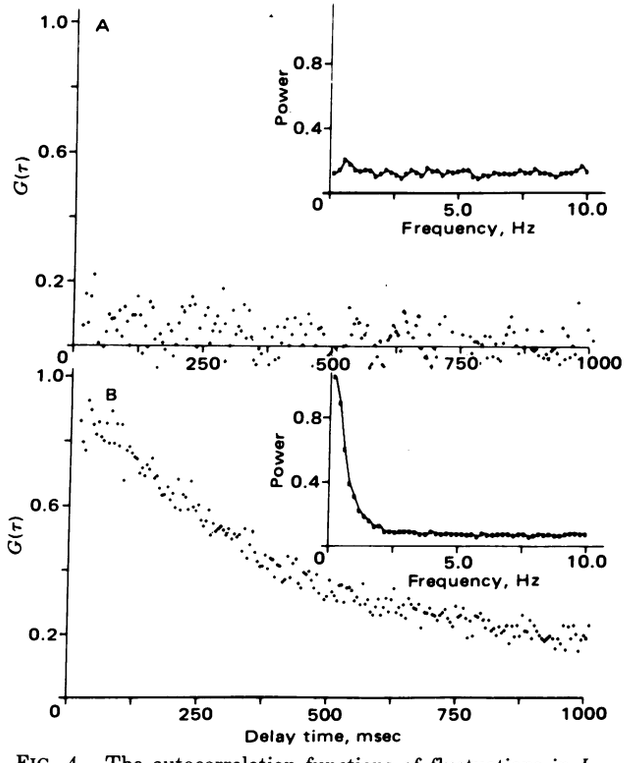

FIG. 4. The autocorrelation functions of fluctuations in $I_{\perp\perp}$ during relaxation (*A*) and during active tension development (*B*). The vertical intercepts are a measure of the number density of cross bridges contributing to the fluctuations. $G(0)/\langle n\rangle^2 = 1/\langle N\rangle$, where $\langle n\rangle$ is the average of the photons detected per bin and $\langle N\rangle$ is the average number of independent fluorescent particles within the beam radius, ω (17). *Insets* show corresponding power density spectra. Vertical scales are in arbitrary units.

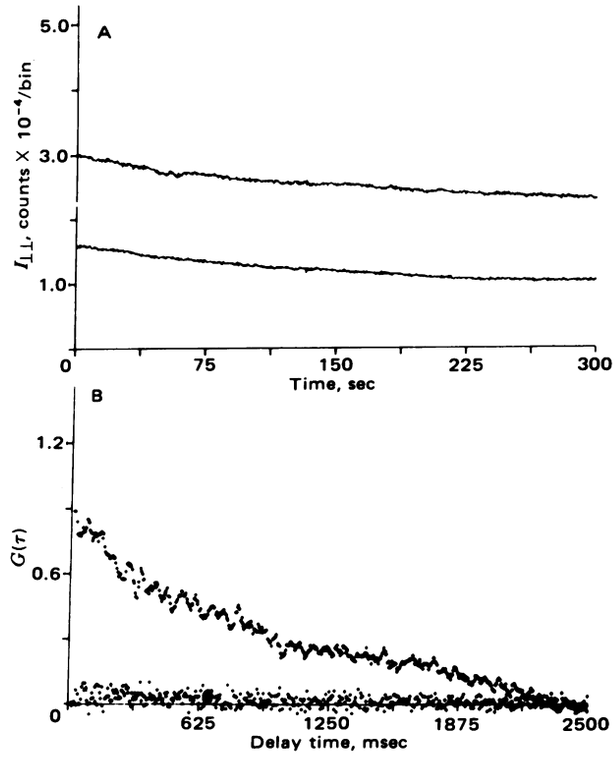

FIG. 5. (*A*) Decay of fluorescence intensity due to photobleaching for a relaxed fiber (upper curve) and for an active fiber (lower curve). (*B*) Autocorrelation function of the fluctuation in $I_{\perp\perp}$ during tension development for a fiber in which myosin has been predominantly labeled (upper curve) and for a fiber in which actin has been predominantly labeled (lower curve). Myosin or actin is labeled depending on whether the fiber is labeled in relaxation or rigor, respectively (22).

RESULTS AND DISCUSSION

$R_\perp$ and $P_\perp$ in Relaxation and in Tension Development. In this work, using 514.5-nm excitation and 580-nm emission, we found in relaxation that $R_\perp = 7.129 \pm 0.130$, and $P_\perp = 0.754 \pm 0.004$. During tension development, $R_\perp = 6.741 \pm 0.160$ and $P_\perp = 0.741 \pm 0.005$. As might be expected from a fluorophore with presumably different optical axes (tetramethylrhodamine), these differ from measurements using IAEDANS (16) or IAF (9). Also, for static states the present measurements are less accurate because the light collection angle in the measuring system is significantly increased to maximize collection efficiency.

Photobleaching. Fig. 3*A* shows decay with time of polarized intensities from an active fiber; the decays in relaxation and rigor are very similar. The quantum yield of photobleaching in all three states is *ca.* 8×10^{-6}. This low value reflects the sturdiness of IATR compared to IAEDANS and IAF. At the expense of reducing S/N we have kept the excitation flux low (4.8×10^{12} photons sec^{-1}). Fig. 3*B* shows that $R_\perp$ and $P_\perp$ suffer no decay with time despite the ongoing photobleaching. These quantities remained constant throughout an experiment, indicating that photobleaching has no directional priference.

Fluctuations in Different Physiological States. The central observations in this work are in Figs. 3, 4, and 6. Fig. 4*A* shows that for a single intensity component such as $I_{\perp\perp}$, $G(\tau)$ and $S(\nu)$ are independent of τ and ν, respectively, when a fiber is at rest. But when the fiber is developing isometric tension, $G(\tau)$ varies with τ and $S(\nu)$ shows that whatever motion produces the fluctuations it is mainly composed of frequencies between 1 and 2.5 Hz (mean half-value of τ, 370 ms). As explained above,

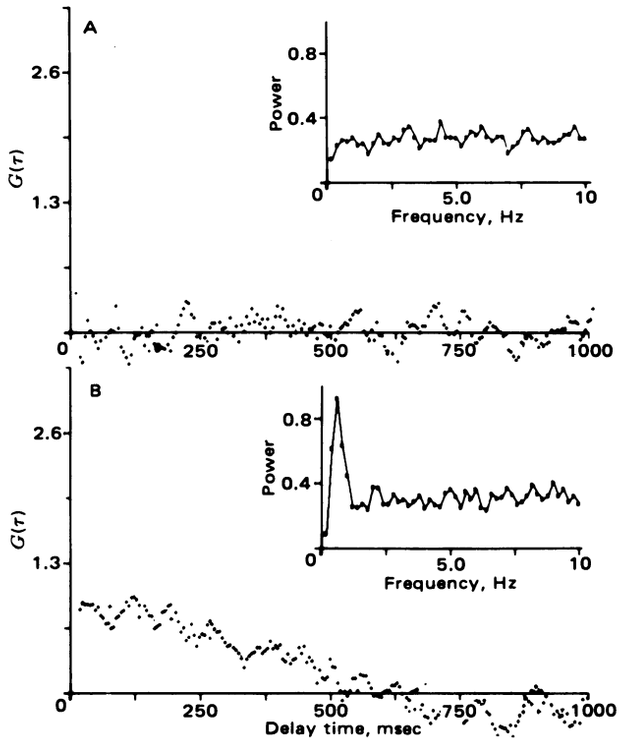

FIG. 6. Autocorrelation functions and power density spectra for $R_\perp$ and $P_\perp$ for a single fiber in relaxation (*A*) and active tension (*B*). One of the intensities ($I_{\perp\perp}$) used in constructing these rational functions is the same as in Fig. 4.

number fluctuations could contribute to these results. But Fig. 6 shows that $R_\perp$, which should be insensitive to number fluctuations, but highly sensitive to rotational motion, behaves the same way—i.e., $G(\tau)$ and $S(\nu)$ indicate that in *a steadily active fiber, but not in the same fiber at rest, there is repetitive rotational motion of the cross bridges,* a motion mainly characterized by frequencies of 1–2 Hz. Sample ATPase frequencies (measured in fibers from the same bundles used in the fluctuation measurements) were 1.6 and 1.9 Hz.

Why Are the Component Frequencies So Slow? From observations on live fibers contracting isotonically we would expect impeller motion to comprise frequencies of 10–20 Hz, not 1–2 Hz. Several factors may contribute to this difference. One is that all events are slower in glycerinated than in live material. Another is that our present detection system tends to miss higher frequencies; to minimize photobleaching we illuminate with a low light flux, which means that bin width must be somewhat long and, thus, that we cannot measure higher frequencies (yet, with 5 ms per bin we should have detected 10–20 Hz). Third is that motion may be slower under isometric tension than in isotonic shortening (3). In time, all of these technical handicaps may be overcome. Even if we are still missing higher frequency motion, however, we emphasize that there is strong evidence for low frequency processes in active, isometrically held, glycerinated fibers, because tension observations (7), ATPase observations (9), and the present observations of rotational cross-bridge motion all point to frequencies in the 1- to 2-Hz range.

Summary and Conclusion. The signals detected in this work originate in fluorophores mainly bound to the cross-bridge moieties. The parameter that we study, a *ratio* of polarized intensities, is insensitive to number fluctuations, but very sensitive to rotational motion. This ratio parameter indicates unambiguously that in steady-state activity (but not in static states such as relaxation or rigor) the bound fluorophores (and presumably the cross bridges) are in repetitive rotational motion. The frequencies of this motion are in the same range as frequencies deduced from tension fluctuations and from ATPase measurements. Several independent experiments have made it unlikely that the fluorescence fluctuations arise from light scattering, birefringence, or number fluctuations. Therefore, our experiments show that tension development is accompanied by repetitive rotational motion of the cross bridges, as would be predicted by the "cycling cross bridge" theory of muscle contraction.

This manuscript was kindly read and constructively criticized by Professors Dan Axelrod, Elliot Elson, George Feher, Douglas Magde, and Robert Pecora. IATR was synthesized by Dr. Marvin Sternfeld. Dr. John Sedat was very helpful to us in our birefringence measurements. The authors gratefully acknowledge the research support of the National Science Foundation, the National Heart, Lung, and Blood Institute, and the American Heart Association. J.B. and M.F.M. are, respectively, Established Investigator and Career Investigator of the American Heart Association.

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