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Observing cycling of a few cross-bridges during isometric contraction of skeletal muscle

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Abstract

During muscle contraction a myosin cross-bridge imparts periodic force impulses to actin. It is possible to visualize those impulses by observing a few molecules of actin or myosin. We have followed the time course of orientation change of a few actin molecules during isometric contraction by measuring parallel polarized intensity of its fluorescence. The orientation of actin reflects local bending of a thin filament and is different when a cross-bridge binds to, or is detached from, F-actin. The changes in orientation were characterized by periods of activity during which myosin cross-bridges interacted normally with actin, interspersed with periods of inactivity during which actin and myosin were unable to interact. The periods of activity lasted on average 1.2 ± 0.4 s and were separated on average by 2.3 ± 1.0 s. During active period, actin orientation oscillated between the two extreme values with the ON and OFF times of 0.4 ± 0.2 and 0.7 ± 0.4 s, respectively. When the contraction was induced by a low concentration of ATP both active and inactive times were longer and approximately equal. These results imply that cross-bridges interact with actin in bursts and suggest that during active period, on average 36% of cross-bridges are involved in force generation.

Introduction

Muscle contraction results from the cyclical interactions of actin and myosin. During this interaction myosin cross-bridges cyclically deliver force impulses to actin. Force, stiffness and ATPase are related to the time average of those impulses (Oplatka 1972). Kinetic constants characterizing contraction can be determined by measuring averages together with the rate constant of force redevelopment (Brenner 1988). Although it is a compelling and successful approach, it involves the application of a specific model (e.g. (Huxley 1957)) of muscle action. A more direct approach is not to have to rely on any specific model. If it was possible to observe a few molecules, any averaging would be easily deconvolved by computing correlation function and the dynamics of a cross-bridge could be directly observed. At the same time it is important to perform experiments *in-* or *ex-vivo*, because muscle proteins are arranged in well-ordered arrays where relative position of actin and myosin are important (Eisenberg et al. 1980). Moreover, the concentration of proteins in muscle is very high (of the order of 100 μ M (Bagshaw 1982)) and at such high concentrations the excluded volume effect come into play. For example, the access to contractile proteins may be limited to only small solvents and as a result certain regions of a muscle cell may become over hydrated and behave differently than isolated proteins in solution (Minton 1981; Minton and Wilf 1981).

Measuring correlation function of a signal, rather than a signal itself, has an important additional advantage: contributions of noise and of autofluorescence signal are significantly reduced. This has been overcome by recent advances in single molecule detection in vitro (Enderlein and Ambrose 1997; Taniguchi et al. 2007; Wang et al. 2007; Willets et al. 2003). In the application to muscle Warshaw and collaborators measured the orientation of a single molecule of smooth myosin II (Quinlan et al. 2005; Warshaw et al. 1998) and Goldman & Selvin et al. measured orientation of a single molecule of myosin V (Forkey et al. 2003; Toprak et al. 2006; Yildiz et al. 2003). Alternative approach, adopted here, is to measure the autocorrelation function of the signal (in the present case: the intensity of polarized fluorescence). The autocorrelation diminishes noise because the white noise is not correlated with itself. Measuring a signal directly is much more difficult. For example, recently we compared the fluorescence lifetime of cardiac myofibrils from healthy and diseased hearts carrying a point mutation that leads to the expression of cardiac hypertrophy phenotype (Mettikolla et al. 2009). We were unable to detect any differences in this signal between healthy and diseased hearts. But when we measured correlation function, we detected significant decrease in myosin kinetics in diseased muscle.

We have chosen to observe actin rather than myosin. Observing actin has five essential advantages. First, labeling actin with phalloidin preserves the regular structure of a myofibril, unlike the less gentle labeling of myosin. Second, phalloidin does not alter the enzymatic properties of muscle (Bukatina et al. 1996; Prochniewicz-Nakayama 1983). Third, phalloidin labels actin specifically and stoichiometrically, which allows strict control of the degree of labeling. Fourth, phalloidin attaches to actin very rigidly because it involves hydrophobic and Van der Waals links. Finally, labeling actin with phalloidin allows observing events that occur only in the area where actin and cross-bridges interact.

This is because in skeletal muscle (in contrast to cardiac muscle) phalloidin initially labels only the ends of thin filaments (Overlap zone, O-band) (Szczesna and Lehrer 1993) – precisely the region where filaments overlap and interact. Observing actin is a valid way of observing the effect of cross-bridges, because it has been known for a long time that actin changes orientation in response to cross-bridge binding (Borovikov Yu et al. 1991; Prochniewicz-Nakayama 1983; Yanagida and Oosawa 1978; Yanagida and Oosawa 1980) and that those changes parallel changes of orientation of a cross-bridge (Borejdo et al. 2004)

The common way to determine kinetics is to follow orientation changes of actin or myosin by measuring polarization of fluorescence (P) of a fluorophore attached to either moiety. P is a sensitive indicator of orientation (Dos Remedios et al. 1972a; Dos Remedios et al. 1972b; Hopkins et al. 1998; Hopkins et al. 2002; Morales 1984; Nihei 1974; Sabido-David et al. 1998; Tregear and Mendelson 1975). For fluorophores in solution, P is defined as the ratio of parallel and perpendicular polarized intensities: $P = (I_V - I_H) / (I_V + I_H)$, where I_V and I_H are the orthogonal intensities of fluorescence obtained using vertical and horizontal polarization of exciting light, respectively. In the case of muscle, where the fluorophores assume fixed orientation with respect to muscle axis, there are two polarizations, $P_H = (I_{HV} - I_{HH}) / (I_{HV} + I_{HH})$ and $P_V = (I_{VV} - I_{VH}) / (I_{VV} + I_{VH})$. Following the original notation of Tregear & Mendelson (Tregear and Mendelson 1975) the first subscript indicates the direction of polarization of the exciting light with respect to the laboratory frame of reference, and the second subscript indicates direction of polarization of the emitted light with respect to the laboratory frame of reference. In this work muscle axis was always vertical (V) and the direction of polarization of the laser was also always vertical (V), so we measured I_{VV} or I_{VH} which allowed us to determine P_V . But in the actual calculations of correlation function we did not use P_V . We used one of the two orthogonal components, either I_{VV} or I_{VH} . The reason for this was that polarized fluorescence is the ratio of two noisy signals. Consequently, its correlation function is noisy and it cannot be carefully analyzed. In the

earlier work we calculated polarization of fluorescence (Borejdo et al. 2007), but it was demonstration of principle, and correlation function was not computed. But to extract kinetic parameters from the data the correlation function must be calculated. We have shown experimentally that over short period of time (where laser instabilities, motion of the microscope etc are minimized) correlation function of one orthogonal component behaves like correlation function of polarized fluorescence (Muthu et al. 2008). Gratton's group demonstrated the same thing theoretically (Barcellona et al. 2004). A good demonstration that correlation function of one component of polarization retains characteristic of correlation of the entire polarization function is a comparison of correlation of one orthogonal intensity (Fig. 3A) with correlation of the corresponding polarization of fluorescence. The comparison (data not shown) reveals that although correlation of polarization retains many essential details, some details (like substructure of the correlation peaks) are lost in the noise.

We were able to select for measurements only a few actin molecules by labeling one in 100,000 actins with rhodamine-phalloidin, and by selecting small detection volume by using confocal detection. The changes in orientation were characterized by periods of activity during which myosin cross-bridges interacted normally with actin, interspersed with periods of inactivity during which actin and myosin were unable to interact. The periods of activity lasted on average 1.2 ± 0.4 s and were separated on average by 2.3 ± 1.0 s. During active period, actin orientation oscillated between the two extreme values with the ON and OFF times of 0.4 ± 0.2 and 0.7 ± 0.4 s, respectively. When the contraction was induced by a low concentration of ATP both active and inactive times were longer and approximately equal.

Materials and methods

Chemicals and solutions

Alexa488 (AP)- and rhodamine-phalloidin (RP) were from Molecular Probes (Eugene, OR). All other chemicals including 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide (EDC), dithiotreitol (DTT), creatine phosphate and creatine kinase were from Sigma. EDTA-rigor solution contained 50 mM KCl, 2 mM EDTA, 1 mM DTT, 10 mM Tris-HCl buffer pH 7.5. Ca-rigor solution contained 50 mM KCl, 4 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT, 10 mM Tris-HCl buffer pH 7.5. Mg-rigor solution contained 50 mM KCl, 4 mM $MgCl_2$, 1 mM DTT, 10 mM Tris-HCl buffer pH 7.5. Contracting solution was the same as Ca-rigor, except that it contained also 5 mM ATP. When low concentrations of ATP were used, *the* contracting solution contained 20 mM creatine phosphate and 10 units/mL creatine kinase (~ 1 mg/mL).

Preparation of myofibrils

Thin strips of glycerinated rabbit psoas muscle were incubated in EDTA rigor solution until they turned white (~ 1 hr). The fiber bundle was then homogenized using a Heidolph Silent Crusher S homogenizer for 20 sec (with a break to cool after 10 s) in Mg^{2+} -rigor solution (it was important that the fibers were not homogenized in the EDTA rigor buffer to avoid foaming). Myofibrils were always freshly prepared for each experiment. Labeled myofibrils (25 μ l) were applied to a coverslip (Menzel-Glaser 20 $\times$ 20 mm #1 or Corning #1 25 $\times$ 60 mm). The sample was left on a coverslip for 3 minutes to allow the myofibrils to adhere to the glass. The bottom cover slip was covered with a small coverslip (to prevent drying) and the two were separated by Avery Hole Reinforcement Stickers. Labeled myofibrils were washed with 5 volumes of the Ca^{2+} -rigor solution by applying the solution to the one end of the channel and absorbing with #1 filter paper at the other end.

Cross-linking

To prevent the shortening of muscle in the contracting solution the myofibrils (1 mg/mL) were incubated with 20 mM EDC for 10 min at room temperature according to procedure of Herrmann (Herrmann et al. 1993). The reaction was stopped by 20 mM DTT. Cross-linking did not affect ATPase (see Fig. 1S of the Supplementary Material). The lack of shortening was checked by comparing the length of a myofibril before and 100 sec after inducing contraction in a TIRF microscope. Within the limits of measuring accuracy on the computer screen (~1%), the length always remained unchanged. The same result was obtained earlier using a confocal microscope (Borejdo et al. 2007). Cross-linked myofibrils are a good model for muscle fiber ATPase and the kinetics of Ca(2+)-activated activity (Herrmann et al. 1994). The large P(i) bursts and kcat values were the same in cross-linked myofibrils and muscle fibers (Herrmann et al. 1993). Those results were confirmed by (Lionne et al. 2003).

ATPase measurements

200 µL of 1 mg/mL myofibrillar suspension was incubated in 0.1 mM ATP for 30, 60, 90 and 120 s. After the specified time, the reaction was stopped by 700 µL of 1 mM HCl. The samples were filtered through a cotton ball in a 1 mL pipette tip. 100 µL of Malachite Green (MG) reagent from the SensoLyte Phosphatase assay kit (AnaSpec, San Jose, CA) was added and incubated for 5 minutes. 10 µL of phosphate contained in the kit was dissolved in 190 µL of deionized water along with 700 µL HCl and 100 µL MG reagent and used as a standard. 1 mL of 10 µM of standard contained 10^{-9} moles of phosphorus. The concentration of phosphate was measured at 650 nm. 200 µL of Ca²⁺- rigor containing 700 µL HCl and 100 µL MG reagent was used as a blank. $[P_i]$ was calculated as $\text{mol}/1 \text{ mol}/\text{min} = \text{Abs}(\text{sample}) * [\text{standard mol}]/\text{Abs}(\text{standard})/[\text{myosin mol}]/\text{minutes}$. The amount of myosin in 200 µL of 1 mg/mL myofibrils was taken as $0.2 * 10^{-9}$ mol. The mean $\pm$ SD of 4 measurements were $3.1 \pm 0.8 \text{ s}^{-1}$ (see Supplementary Material Fig. 1S).

Labeling

1 mg/mL myofibrils (~ 4 µM actin) were mixed with 0.1 nM Alexa488-phalloidin+10 µM unlabeled-phalloidin or with 0.1 nM rhodamine-phalloidin+10 µM unlabeled-phalloidin. Unlabeled phalloidin was necessary to prevent uneven labeling. If it was not there, the sarcomeres closest to the tip of the pipette used to add the label would have contained more chromophores than sarcomeres further away from the tip. The degree of labeling was 10 µM/0.1 nM = 100,000, i.e. on the average 1 actin protomer in 10^5 was fluorescently labeled.

Data collection

The experiments were done using Micro Time 200 (PicoQuant GmbH, Berlin, Germany) confocal system coupled to Olympus IX 71 microscope. The objective was water immersion NA=1.2, 60×. The excitation was by a 470-nm laser pulsed diode, and the observation was through a 500-nm long pass filter. The confocal pinhole was 30 µm. The instrument measured fluorescence lifetimes as well as anisotropies. Whenever indicated, the data was collected by ISS-Alba-FCS (ISS Co, Urbana, IL). The excitation was by a 532 nm CW laser. The confocal pinhole was 50 µm. Fluorescence was collected every 10 µs. Orthogonally linearly polarized analyzers were placed before Avalanche PhotoDiodes (APD's). The laser was polarized vertically (on the microscope stage). The myofibrils were also vertical.

Data analysis

The signal was smoothed by adding photons over small time intervals. The smoothed signal was fitted to an exponential that was subsequently subtracted from the signal. From this the autocorrelation function was calculated. The autocorrelation function was fitted to a train of triangular waves by a least squares fit. It was assumed that the “hidden signal” to look for

was a rectangular wave. The programming and calculations were done in Matlab. The autocorrelation functions were calculated in the Fourier domain by taking the Fast Fourier transform of the signal padded with an equal number of zeros in order to not have the last points in the signal to correlate with the first.

Measuring anisotropy in solution

Fluorescence anisotropies were measured by time-domain technique using FluoTime 200 fluorometer (PicoQuant, Inc.). The excitation was by a 475-nm laser pulsed diode, and the observation was through a monochromator at 590 nm with a supporting 590-nm long wave pass filter. The FWHM of pulse response function was 68 ps (measured by PicoQuant, Inc.). Time resolution was better than 10 ps. The intensity decays were analyzed in terms of a multi-exponential model using FluoFit software (PicoQuant, Inc.).

Rotation of Rhodamine-phalloidin bound to F-actin

For the quantitative measurement of orientation, it is important to know whether the probe is immobilized by the protein so that the transition dipole of the fluorophore reflects the orientation of the protein. For this reason we compared *the* decay of *the* anisotropy of RP and of RP on F-actin. The decay of *the* anisotropy of RP was best fitted by a single exponent. 100% of the signal decay was contributed by the decay time of 0.519 ns, consistent with *the* rotation of a molecule with $M_w=1,250$. No independent rotation of rhodamine moiety was observed. The decay of anisotropy of RP coupled to thin filaments was best fitted by the two exponents with correlation times of 0.665 and 36.8 ns with the relative contributions of 13.7 and 86.3%, respectively. The short correlation time is due to the rotation of rhodamine moiety independent of phalloidin moiety which remains bound to F-actin. It is not due to rotation of free rhodamine-phalloidin, because binding of phalloidin to F-actin is extremely strong. The long correlation time is due to rotation of F-actin oligomers. Thus over 86% of fluorescent phalloidin is immobilized by F-actin. This is consistent with the fact that *the* probes attached to proteins through interactions that stretch over large surface areas, such as hydrophobic or Van der Waals interactions are attached more rigidly than probes that are attached by covalent links.

Results

Imaging

A typical lifetime image of a rigor myofibril is shown in Fig. 1A. (Lifetime image is shown here because it was superior to conventional intensity image. The fluorescent lifetime and the decay of fluorescent intensities associated with this image are shown in Supp. Mat. Fig. 2S). The various bands are best identified with the aid of image of myofibrils more heavily labeled with phalloidin (10 nM RP + 10 μ M UP; Fig. 1C). This image clearly shows that that there are two bright overlap bands (O-bands) in a center of each sarcomere (i.e. fluorescence does not originate from the I-bands) and that each is separated by a dark I-band and H-zone. This is consistent with earlier findings that phalloidin originally labels the ends of actin filaments (Szczesna and Lehrer 1993). After this initial binding phalloidin redistributes itself to the I-band, a process which takes several hours (Ao and Lehrer 1995). As a result, only those actin protomers that are located in the region where interactions with myosin occur, are initially labeled.

Number of detected actin molecules

The red circle in Fig. 1A indicates the projection of the confocal aperture on the image plane. The diameter of this projection (0.5 μ m) is equal to the diameter of the confocal pinhole (30 μ m) divided by the magnification of the objective (60 $\times$). Fig. 1B shows the time

course of the parallel polarized signal (I_V)* collected from the Detection Volume (DV). The trace exhibits a classical symptoms of bleaching of a few molecules - a sudden stepwise drop of intensity to the background level. The major part of a decline, attributed to bleaching of a single molecule, consisted of a decrease in the vertical component of the intensity fluorescence rate (δI_V) of $\sim 1,000$ photons/s. The perpendicular component (I_H) of the signal was ~ 2 photons/ms. The total loss of fluorescence rate due to bleaching of a single molecule was $\delta I_V + \delta 2 \cdot G \cdot \delta I_H \approx 3$ photons/ms, where G is the correction factor ($=1.06$). This number carries significant uncertainty. The time course and the time before photobleaching were different for each spot. This is not surprising because different fluorophores reside at different distances from the focus of the illuminating laser beam. They are thus subjected to different illuminating light intensities and take varying amount of time to absorb the number of photons required for photobleaching. Overall, a single fluorophore in rigor muscle contributed photons at a rate of 3 – 10/ms.

Assuming that adding ATP (i.e. inducing contraction) does not alter the quantum yield of rhodamine, the knowledge of the photon rate makes it possible to estimate the number of detected molecules during contraction: Fig. 2A shows a typical signal of contracting muscle. The perpendicular (I_H) and parallel (I_V) intensities are shown in the red and blue, respectively. The decay of the signal during the first 4 s is due to photobleaching of some of the fluorophores. The contributions of these fluorophores were rejected in analysis, and the data was analyzed only when the signal reached steady-state (4–10 s range in this case). The fluorophores that remained unbleached after 4 sec of illumination cannot be bleached within 20–30 s. The average counts from I_V and I_H channels after 4 s were 4.4 and 1.8 counts/ms, respectively. The inset to Fig. 2A shows the signal from an empty area immediately adjacent to a myofibril. The average counts from I_H and I_V channels of background were $I_{Hb} = 0.4 \pm 6$ and $I_{Vb} = 0.7 \pm 9$ photons/ms, respectively. The total photon rate during 4–10 s interval was $I_{Tot} = (I_V - I_{Vb}) + 2 \cdot G \cdot (I_H - I_{Hb}) = 9.8$ photons/ms. This is consistent with a contribution of 1–3 molecules of actin.

We wish to point out that another way to determine the number of fluorophores in the DV is to use the well known dependence of the maximum value of correlation function, $G(0)$, on the number of molecules N : $G(0) = 1 + 1/N$. The data presented in the Appendix is consistent with the conclusion that we observe only few fluorophores.

Autocorrelation of polarized fluorescence of contracting muscle

In principle, when a single molecule is observed, it should be possible to distinguish individual impulses by inspection of the intensity traces. In practice this was not possible because of the noise. Fig 2B shows I_{VH} intensity trace during steady-state on a magnified scale. Any periodicity is lost in a noise. A standard way to reduce the contribution of white noise to a periodic signal is to compute its autocorrelation function. The correlation function at a given delay time τ is the sum of the products of a signal multiplied by a signal shifted by a delay time τ . Thus if τ is small the correlation function will be large. If τ is equal to the period of a signal, the product will also be large because there is correlation between the signal and its value one period later. But the situation is different with white noise. Now the only correlation present is between the initial value and the value a short time τ later. As τ increases, a high frequency white noise assumes the value of the opposite sign to that of the original point. The product of such pairs will be negative and the sum of the products will be smaller. If τ is sufficiently large, the sum will be zero. The relation between the signal, correlation function and its power spectrum is shown in Fig. 2C. The autocorrelation

* As mentioned above, the muscle axis and the direction of polarization of the laser were always vertical (parallel), so in this paper we abbreviate I_{VV} with I_V and I_{VH} with I_H .

corresponding to the signal of Fig. 2B is shown in Fig. 2D (the corresponding power spectrum is shown in Supp. Mat. Fig. 3SA). The periodicities are now clearly visible.

Inspection of Fig. 2D reveals that the correlation function contains two types of periodicities: a slow one, characterized by a sudden bursts of activity every couple of seconds, and a fast one, characterized by rapid bursts within the main peaks. Fig. 3 shows a typical example of an experiment which was analyzed for slow oscillations. **A** shows the original signal from the contracting muscle. **B** is a negative control, showing no activity whatever in rigor muscle. The same negative result was obtained from non-crosslinked myofibrils bathed in contracting solution that were prepared from fibers that were pre-stretched beyond overlap in a relaxing solution. The correlation functions are shown in **C** & **D** (the corresponding power spectra are shown in Supp. Mat. Fig. 4S). The red line in Fig. 3C shows the least square fit of the slow oscillations to a piecewise linear train of triangles. The reason it is fit to a series of triangles is that a waveform which gives rise to triangular correlation function is a train of rectangular waves. The train of rectangular waves is the simplest time course of orientation change during muscle contraction. We recognize that a simple ON-OFF mechanism is an oversimplification of the actual events (Houdusse and Sweeney 2001), but there is no doubt that the main cyclical events giving rise to orientation change (binding and dissociation of a myosin cross-bridge) are correctly represented (see Discussion). The binding of a cross-bridge most likely leads to a reduction in the number of the bending modes of actin rather than the reorientation of the actin monomer, because the work of Yanagida and collaborators implied the lack of any gross rotational motion of a monomer (Yanagida and Oosawa 1978; Yanagida and Oosawa 1980)(Fig. 7B). Conversely, the dissociation of a cross-bridge probably leads to the increase in the number of bending modes. The time during which the signal is high and low indicates the ability or inability of cross-bridges to access thin filament. We call these times t_A and t_I . t_A was extracted by measuring the time from 0 until the correlation function first changed slope. t_I was measured from the time the correlation function changed slope to the next maximum. These times are tabulated in Table 1 for all 17 independent experiments on 6 different myofibrillar preparations.

Fig. 4 shows a typical example of an experiment which was analyzed for fast oscillations. **A** and **B** show the original signal. The correlation functions are shown in **C** & **D** (the corresponding power spectra are shown in Supp. Mat. Fig. 5S). The green line in **C** shows the fit of the fast periodicities in the experimental correlation function to a piecewise linear train of triangles. The time during which the signal is high and low indicates the ability or inability of cross-bridges to bind the thin filament. When a cross-bridge strongly attaches to actin the anisotropy is high because during this time the rotational freedom of a whole actin filament is low. The opposite is true for detached cross-bridge. We call these times t_{ON} and t_{OFF} . Fig. 5 demonstrates how the times were extracted from the correlation function. t_{ON} was extracted by measuring the time from 0 until the correlation function first changed slope. t_{OFF} was measured from the time the correlation function changed slope to the next maximum. The inset shows the autocorrelation function with X-axis plotted on log scale. These times, measured at high (5mM) ATP, are summarized in Table 1.

Contraction induced by low [ATP]—It is known that in the presence of low [ATP] the rate of actin motion in the in vitro motility assay is low (Baker et al. 2002) and that the decrease in the concentration of ATP causes the duration of the strongly bound state of skeletal myosin to increase (Baker et al. 2002). Fig. 6A shows a typical record of the time course of polarized fluorescence of a myofibril whose contraction was induced by 10 μ M ATP. The autocorrelation function is shown in Fig. 6B. It is clear that the major change was an increase in t_A and t_I . We carried out 10 experiments using 10 μ M ATP to induce

contraction. The average value of t_A and t_I are given in Table 1. The differences in means are statistically significant for both times ($t=7.03$, $P<0.001$ for t_A and $t=2.30$, $P=0.042$ for t_I).

Discussion

We've used fluorescence polarization to measure the kinetics of the binding of myosin cross-bridges to actin. Anisotropy is a ratio of two noisy signals and its autocorrelation function is very noisy. We have therefore measured the correlation of one of its intensity components. This is justified, because we have previously shown that parallel and perpendicular fluorescence intensities behave similar to anisotropy (Muthu et al. 2008). Moreover, as already mentioned, Barcellona et al. (Barcellona et al. 2004) have shown that the autocorrelation function of P approximates the autocorrelation function of polarized intensity.

The kinetics was measured from a few molecules of actin. Although there were originally about 3–10 molecules in the DV, as shown in Fig. 1A, the majority of fluorophores become bleached out. Few fluorescent molecules remaining after bleaching out could not be destroyed in the several seconds (the remaining molecules are probably located further from the focus of the laser beam than the bleachable ones). This fitted our purpose well, because it gave rise to undiminished fluorescence for at least 10–20 s necessary to perform the experiment.

It should be noted that the exact number of detected molecules is unimportant in determining kinetics, as long as the number falls within mesoscopic regime, i.e. stochastic fluctuations become important (Qian et al. 2002). The size of fluctuations is largest when one molecule is observed (Elson and Magde 1974), but the autocorrelation function is the same whether 1 or 10 molecules are observed.

Not surprisingly, a signal from a single molecule was very noisy - it originated from only a few actin molecules among 200,000 present in a Half Sarcomere (HS). To reduce noise, we constructed the correlation function of the signal. Correlation function reduces the white noise because there is no correlation between the noise at a given time and the noise any time later. This reduction comes at a heavy price - the degeneracy of the correlation function. The phase information is lost by squaring the amplitude power spectrum (or, in the case where correlation is computed directly, by multiplying signal by itself). Fig. 7A illustrates the fact that there are many waveforms giving the same correlation function. For example, crystallographic studies, single-molecule studies, spectroscopic experiments, and X-ray diffraction experiments on muscle fibers suggest that in addition to ON and OFF conformations, cross-bridge lever arm assumes an additional -- weak binding -- conformation (Reedy 2000; Sweeney and Houdusse 2004; Takagi et al. 2004). This leads to a conclusion that polarized intensity of actin monomer assumes 3 different values, as illustrated in the bottom left column of Fig. 7A. If the duration of each step were equal, the result would lead to a correlation function of the same shape as 2-step process. Because of this degenerative property of the Fourier Transform, our results cannot provide evidence for such a state.

This degeneracy makes it necessary to presume a shape of the signal giving rise to the observed correlation function. The simplest signal giving rise to the observed correlation function (a linear train of triangles) is a linear train of rectangles. As we said earlier, this is an oversimplification, but the one that most likely correctly reflects the main events occurring during contraction. It is interesting to note that the correlation function is comprised of two periodic processes. They both give rise to a rectangular signal of the same shape, but with a different period (Fig. 5B). The first, a slow process, probably reflects the

ability of a cross-bridge to bind to actin. The period of this process was 3.5 s. It implies that cross-bridges bind to actin in bursts. During the time t_I no binding occurs. During this time the cross-bridges may be unable to reach actin because they may have restricted access or because their axial position does not allow the power stroke to take place. During the time t_A cross-bridges undergo normal interaction with actin. The cycle of events is illustrated in Fig. 7C. Fig. 7D shows the assumed shape of the corresponding correlation function: Cross-bridges carrying the products of the hydrolysis of ATP bind strongly to actin leading to a decrease in the number of bending modes of the thin filament and presumably resulting in high anisotropy. The dissociation of Pi leads to force generation followed by the dissociation of ADP and the formation of rigor complex. The attached state ends when the myosin cross-bridge binds to a new molecule of ATP. The strongly attached state lasts t_{ON} seconds. The cross-bridge detachment results in increase in the number of the bending modes of thin filament and leads presumably to a low anisotropy. This state lasts t_{OFF} seconds. The average cycle time determined in the present work $t_C = t_{ON} + t_{OFF} = 1.1$ s, is longer than determined from the maximal ATPase activity measured in isometric myofibrils here (~300 ms) and in glycerinated muscle fibers (120–100 ms, (Hilber et al. 2001)). This is most likely caused by the fact that the ATPase measurements in muscle fibers are not equivalent to measurements of changes in the orientation of the phalloidin-actin protomer. The former reflects a gross phenomenon, while the SMD polarization measurements report on a local orientation change. It is likely that a hydrolysis event by a distant cross-bridge is not sensed by the actin molecule under observation. The binding signal propagates only over a few actin monomers (Ando 1989) and the nearest cross-bridge on the same actin filament is 38.5 nm away.

Duty ratio

A useful measure of the myosin cross-bridge kinetics is the duty cycle - a fraction of the total cycle time that a cross-bridge remains attached to actin. A cross-bridge is in a strongly attached state for a period of time t_{ON} , while the total cycle time is $t_I + t_A$. The duty ratio of the entire isometric cycle, $\Psi = t_{ON}/(t_I + t_A)$ was ~11%. This is smaller than data obtained earlier by Cooke et al (Cooke et al. 1982) by electron spin resonance, and by Duong and Reisler (Duong and Reisler 1989) by measuring tryptic digestion at the 50/20 kD junction of the myosin heavy chain. The value of Ψ obtained here is also smaller than the value of ~0.3 obtained by Cooper et al. (Cooper et al. 2000). A different measure of the activity of cross-bridges is the “accessible” duty cycle - a fraction of the accessible time that a cross-bridge remains attached to actin. This ratio was $\Psi' = t_{ON}/(t_{ON} + t_{OFF}) = 36\%$.

The most obvious change in the pattern of contraction induced by low concentration of ATP was in t_A and t_I . The values of t_A and t_I increased 160 and 40%, respectively. The entire kinetics of contraction became different, as demonstrated by the fact that Ψ became now ~50%. These results are consistent with the earlier results (Baker et al. 2002) on isolated single myosin molecules in vitro.

Relaxed muscle

We did not systematically analyze relaxed muscle. The reason was that in a few experiments the signals from active and relaxed muscle were similar. This was because cross-linking caused damage to the regulatory proteins. Herrmann et al. (Herrmann et al. 1993) estimated that ~8% of the myosin cross-bridges were cross-linked to actin using 2 mM EDC. This was apparently sufficient to permanently turn on the system. The problem is even more severe in our case because 2 mM EDC was not sufficient in our hands to completely prevent shortening. We had to increase the concentration of EDC to 20 mM to assure that no shortening whatever occurred. With such high [EDC], the degree of cross-linking (in heart papillary muscle) was 30% (Mettikolla et al. 2009). We conclude that the extensive cross-

linking that we needed to employ to completely eliminate shortening, permanently turned on the system and made experiments on relaxed muscle not reliable.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

t_I	the time cross-bridge is unable to interact with actin
t_A	the time cross-bridge is able to interact with actin
t_{ON}	the time cross-bridge is strongly attached to actin
t_{OFF}	the time cross-bridge is detached from actin
Ψ	Duty Cycle of the cross-bridge
AP	Alexa488-Phalloidin
RP	Rhodamine-Phalloidin
UP	Unlabeled-Phalloidin
DV	Detection Volume
EDC	1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide
DTT	Cleland's reagent
APD	Avalanche Photodiode
FCS	Fluorescence Correlation Spectroscopy
DV	Detection Volume
HS	Half Sarcomere

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Appendix

The alternative way to determine the number of fluorophores in the Detection Volume by using properties of correlation function is to use the well known dependence: 1

$$G(0)=1+\frac{1}{N}$$

where $G(0)$ is the value of the correlation function at time 0 and N is the number of molecules. The conventional correlation function used in FCS is the correlation of a number of uncorrelated fluorophores entering and exiting the detection volume, thereby contributing to the detected signal. The contribution may be large or small, depending on whether *the* molecule enters the volume near its center or near its periphery, but it always begins with *the* contributing signal being zero (except the shot noise). In contrast, the correlation function that concerns us here is slightly different. There may be a number of fluorophores contributing *to* the uncorrelated signals, but these contributions are never zero, they just oscillate between the two levels depending on the instantaneous orientation state of the molecule. An example of such a signal is shown below:

Here u denotes the signal from one fluorophore with average $\langle u \rangle$, and δu denotes the difference of the two levels, i.e. the span of the signal. Also, since the time span of the signal is rather short the correlation at the longer times has less data to correlate and then seems lower. Therefore the normalized correlation function is calculated here by first subtracting a fitted exponential, which is almost constant, calculate the correlation function and then divide by the correlation function calculated in the same way *as* the fitted exponential. The normalized correlation function of Fig. 3 would look like this:

It follows from the normalization that $G(0) = 1.016$.

The correlation function is then $\langle u \cdot u \rangle$, and normalized correlation function $G = \langle u \cdot u \rangle / \langle u \rangle^2$. Assume the signal is from a number of fluorophores, say N . Then the total signal is:

$$u = \sum_{n=1}^N u_n. \text{ Assume all fluorophores are equal, but uncorrelated. Then } \langle u \rangle = N \langle u_1 \rangle \text{ and } \langle u \cdot u \rangle = N \langle u_1 \cdot u_1 \rangle + N(N-1) \langle u_1 \rangle^2.$$

The normalized correlation function is then:

$$G = 1 + \frac{1}{N} \frac{\langle u_1 \cdot u_1 \rangle - \langle u_1 \rangle^2}{\langle u_1 \rangle^2}$$

But at time zero $\langle u_1^2 \rangle - \langle u_1 \rangle^2 = \langle \delta u_1^2 \rangle$. So then the correlation function at time zero is:

$$G(0) = 1 + \frac{1}{N} \frac{\langle \delta u_1 \rangle^2}{\langle u_1 \rangle^2}$$

This means that we cannot determine the number of fluorophores and the span of the signal of a fluorophore at the same time from a single $G(0)$. However, given that $G(0) - 1 \approx 0.016$, $\delta u/u$ can readily be calculated for a given N (taking care that $u_I = 2u$). The table below shows different possibilities that can be extracted from the present data.

N	$\delta u/u$
1	0.2530
2	0.3578
3	0.4382
4	0.5060
5	0.5657
10	0.8000
15	0.9798
20	1.1314

In the data presented in Fig. 3, the average signal was 43 photons/10 ms and its SD 12 photons/10 ms. $\delta u/u = 0.279$, confirming the earlier suggestion that a signal was contributed by a single molecule.

Fig. 1.

(A): The image of a myofibril in rigor sparsely labeled with fluorescent phalloidin. The location of various bands is indicated by white arrows. The red arrow points to the area ($0.5 \mu\text{m}$ in diameter) from which the microscope collects the data. Myofibril irrigated with 0.1 nM Alexa488-phalloidin + $10 \mu\text{M}$ unlabeled phalloidin. The decay of fluorescent intensity of the O-band is shown in Fig. 2S of the Supplementary Material. (B): Sudden drop of I_y intensity to the level of the background in rigor muscle – behavior characteristic of single molecule bleaching. The rate of arrival of fluorescent photons was estimated as $4/\text{ms}$ (see text). (C): Lifetime image of a myofibril irrigated with higher concentration of the dye (10 nM Alexa488-phalloidin + $10 \mu\text{M}$ unlabeled phalloidin) to pinpoint location of fluorescence in different bands more clearly. Cross-linked myofibril, excitation at 470 nm , emission viewed through 500 nm long-pass filter. Laser power $2 \mu\text{W}$. $60\times$, $\text{NA}=1.2$ water immersion objective.

Fig. 2.

(A): The time course of polarized intensity of contracting myofibril. Myofibrillar axis is Vertical on the microscope stage. Laser polarization is Vertical on the microscope stage. Counts in H channel (red) and V channel (blue) are the fluorescence intensities polarized perpendicular and parallel to the myofibrillar axis, respectively. Inset: Signal from an empty area immediately adjacent to the myofibril. Excitation 470 nm, emission >500 nm. Laser power = 2 μW , light flux $\sim 7 \mu\text{W}/\mu\text{m}^2$. (B): The same signal with the first 6.5 s removed. Note that the vertical scale is magnified 10 $\times$. The fluctuations during 6.5–10 s are contributed by only a few molecules of actin. (C): The schematic representation of a relation between signal, correlation function and power spectrum. Amplitude power spectrum is a Fourier Transform (FT) of the signal. Absolute square of amplitude spectrum is the power spectrum, whose Reverse Fourier Transform (RFT) is the correlation function. In practice one computes correlation function from signal by first computing Amplitude and Power Spectrum. (D): the autocorrelation function of the signal in B. The intensity data was fitted to the exponential function, the exponential was subtracted from the data to get zero average value and correlation function was computed. In order to get correlations only between corresponding points, padded zeros have been added before the correlation calculation.

Fig. 3.

(A): The time course of polarized intensity of contracting myofibril. Counts in horizontal channel1 (red) and vertical channel2 (blue) are the fluorescence intensities polarized perpendicular and parallel to the myofibrillar axis, respectively. Vertical axis -counts during 1 ms. (B): The time course of polarized intensity of rigor myofibril. (C): The autocorrelation function of counts in channel 1 of for the signal in A. Red line - the best fit of the experimental autocorrelation function to piecewise triangular waveform. (D): the autocorrelation function of rigor muscle (the signal in B). Myofibrillar axis is Vertical on the microscope stage. Laser polarization is Vertical on the microscope stage. Excitation 470 nm, emission >500 nm. Laser power = 2 μW , light flux $\sim 7 \mu\text{W}/\mu\text{m}^2$. Data collected with PicoQuant MT200.

Fig. 4.

Example of a signal which gives correlation function with clear substructure in slow oscillating peaks. The time course of polarized intensity of contracting (**A**) and rigor (**B**) myofibril. Vertical axis - counts during 1 ms. The autocorrelation function of counts in channel 1 (**C**). Green line - fit of the experimental autocorrelation function to piecewise triangular waveform. The autocorrelation function of rigor muscle (**D**). Data collected with ISS Alba.

Fig. 5.

A. The autocorrelation function was fitted by a slowly oscillating (red) and rapidly oscillating (green) train of triangles. t_A and t_{ON} are defined as times from the peak of the slow or rapid triangles to zero, respectively. t_I and t_{OFF} are the times remaining to complete a cycle. The inset shows the autocorrelation function with X-axis plotted on log scale. The initial decay is characteristic of rapid oscillations. **B** - the signal corresponding to the autocorrelation function in A.

Fig. 6.

A. The raw signal from muscle contracting in the presence of 10 μ M ATP. Ch1 (red) and ch2 (blue) are the fluorescence intensities polarized perpendicular and parallel to the myofibrillar axis, respectively. The number of detected molecules in low [ATP] experiment is estimated from the intensity of the signal as before: average of I_V and I_H signal: 117 and 69 photons/66ms. Total signal= $I_V + 2 \cdot G \cdot I_H = 255$ photons/66 ms = 3.8K photons/s. Background = 1.9K photons/s. The signal originates from ~1–3 fluorophores. **B** - The correlation function of the blue signal. Data was fitted to the exponential function, the exponential was subtracted from the data to get zero average value and correlation function was computed. The red line is the least square fit to the correlation. In order to get correlations only between corresponding points, padded zeros have been added before the correlation calculation. 1000 points averaging was used. Excitation 532 nm, emission > 590 nm. The data was collected every 10 μ s. The vertical scale is the number of counts during 66.7 ms. Myofibrillar axis is vertical on the microscope stage. Laser polarization is vertical on the microscope stage. Data collected with ISS Alba.

Fig. 7.

A. Examples of degeneracy of autocorrelation function. Many waveforms have the same autocorrelation function. The relationship between molecular events (**B**), the observed signal calculated from the autocorrelation function (**C**). The fact that anisotropy is high when myosin is strongly bound to actin and low when it is dissociated from it is an assumption. (**D**) correlation function.

Accessible-Inaccessible and ON-OFF times obtained from contractions induced by high and low ATP concentrations. N is the number of independent experiments.

Table 1

[ATP]	t_A (s)	t_I (s)	N	t_{ON} (s)	t_{OFF} (s)	N
5 mM	1.2 ± 0.40	2.3 ± 1.0	17	0.4 ± 0.2	0.7 ± 0.4	10
10 μ M	3.1 ± 0.7	3.2 ± 0.7	11	-	-	10