PHOSPHORYLATION OF MYOSIN REGULATORY LIGHT CHAIN HAS MINIMAL EFFECT ON KINETICS AND DISTRIBUTION OF ORIENTATIONS OF CROSS-BRIDGES OF RABBIT SKELETAL MUSCLE


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Running Title: Degree of order and kinetics of phosphorylated (P) and de-P cross-bridges.

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ABBREVIATIONS

ACF – Autocorrelation Function
DV - Detection Volume
FLCS - Fluorescence Lifetime Correlation Spectroscopy
FWHM - Full Width at Half Maximum
HS - Half Sarcomere
LC1 - Myosin Light Chain 1
MBP-C - Myosin Binding Protein-C
MHC - Myosin Heavy Chain
MLCK - Myosin Light Chain Kinase
PF - Polarization of Fluorescence
PSF - Point Spread Function
RLC - Regulatory Light Chain of Myosin
SD -- Standard Deviation
S/N - Signal-to-Noise Ratio
SRX - Super Relaxed State
XB -- Cross-Bridge
ABSTRACT

Force production in muscle results from ATP-driven cyclic interactions of myosin with actin. A myosin cross-bridge consists of a globular head domain, containing actin and ATP-binding sites, and a neck domain with the associated Light Chain 1 (LC1) and the Regulatory Light Chain (RLC). The actin polymer serves as a "rail" over which myosin translates. Phosphorylation of the RLC is thought to play a significant role in the regulation of muscle relaxation by increasing the degree of skeletal cross-bridge disorder and increasing muscle ATPase activity. The effect of phosphorylation on skeletal cross-bridge kinetics and the distribution of orientations during steady-state contraction of rabbit muscle is investigated here. Since the kinetics and orientation of an assembly of XBs can only be studied when an individual XB makes a significant contribution to the overall signal, the number of observed XBs was minimized to ~20 by limiting the detection volume and concentration of fluorescent XBs. The autofluorescence and photobleaching from an ex vivo sample was reduced by choosing a dye that was excited in the red and observed in the far red. The interference from scattering was eliminated by gating the signal. These techniques decrease large uncertainties associated with determination of the effect of phosphorylation on a few molecules ex-vivo with millisecond time resolution. In spite of the remaining uncertainties we conclude that the state of phosphorylation of RLC had no effect on the rate of dissociation of cross-bridges from thin filaments, on the rate of myosin head binding to thin filaments and on the rate of power-stroke. On the other hand, phosphorylation slightly increased the degree of disorder of active cross-bridges.
**INTRODUCTION**

Ca\(^{2+}\) binding to thin filaments is a major factor contributing to the initiation of skeletal muscle contraction. But it is thought that in addition to Ca\(^{2+}\) another factor, phosphorylation of the Regulatory Light Chain (RLC) of myosin, may play an important regulatory role. Phosphorylation of skeletal myosin RLC is accomplished by skeletal myosin light chain kinase (MLCK) with Ca\(^{2+}\)-calmodulin acting as a co-enzyme. A single twitch or short tetanus induces a small amount of phosphorylation of RLC (10). Phosphorylation and phosphatase-induced de-phosphorylation of RLC assure that the level of phosphorylation during the steady-state contraction is relatively constant. It is well known that the level of phosphorylation increases during heavy use and fatigue (10, 26). The current thinking is that RLC transmits the influence through myosin heavy chain (MHC) and by stabilizing the lever arm. It has been suggested that phosphorylation of RLC may cause charge repulsion with the thick filament core in a heart (46, 72) and thus move heads closer to thin filaments. Moving heads closer to actin would increase the likelihood of MHC interacting, which in turn is expected to increase the rate of force development. Indeed, Metzger et al. have shown that phosphorylation of RLC in skeletal muscle increased the rate of tension redevelopment at lower [Ca\(^{2+}\)] but was unaffected at maximum [Ca\(^{2+}\)](49). The same was true in in vitro studies of isolated striated (38, 72) thick filaments. Colson et al. (9) used synchrotron low-angle X-ray diffraction to show that phosphorylation of either RLC or cMyBP-C resulted in displacement of XBs away from the thick filament backbone, in cardiac muscle. The other effect of phosphorylation of RLC is the stabilization of the lever arm, which would make it easier to transmit force (63).

Ca\(^{2+}\) withdrawal from myofilament space is a major contributing factor in relaxation (24). It has been shown to influence order and ATPase of XBs in relaxed muscle (67). De-phosphorylated muscle having low ATPase activity and high degree of XB order has been suggested to exist in a new super-relaxed state (SRX) (11). It applies to both skeletal (67) and cardiac muscle fibers (31). We have recently confirmed and quantified these findings by examining the local order of a few XBs in a relaxed half-sarcomere (51).

In this communication we examine the influence of RLC phosphorylation on the movement and distribution of the orientations of XBs during contraction at the level of individual molecules. There are three fundamental reasons for examining contraction using a small number of XBs:

Steady-state kinetic information about XBs can be obtained from fluctuations of orientations of XBs (in our case, the polarization of fluorescence). The relative size of fluctuations is large only when the number of XBs
is sufficiently small (22, 23). This allows determination of the kinetics of contraction by the so-called "mesoscopic" approach, first applied to biological problems by Elson et al. (22, 23). The reason why it is necessary to observe only a small number of molecules at any time is fully explained in (51). In our experiments a typical number of molecules was 20, and the corresponding fluctuation was ~22%. The use of small number of molecules eliminates problems associated with non-uniformity of contracting sarcomeres (74), as fully explained in (51). Finally, our method recognizes that only when an individual XB makes a significant contribution to the overall signal, i.e. the total number of XBs is small, its contribution can significantly affect the total distribution of orientations.

Since the kinetics and orientation of an assembly of XBs can only be studied when an individual XB makes a significant contribution to the overall signal, we minimized the number of observed XBs by limiting the detection volume and concentration of fluorescent XBs. The ideal number of molecules to observe is $N=1$, because then 100% of the signal carries kinetic information\(^1\). We have chosen to investigate ~20 molecules, because in our hands with current technology the signal from a single molecule in an \textit{ex vivo} sample is not enough to collect data with millisecond time resolution and overcome complications due to light scattering and autofluorescence.

There has been many measurements of the dynamics of single motor proteins \textit{in vitro} because they do not suffer from complications due to high protein concentration, light scattering, autofluorescence and the presence of physical barriers which are encountered by motor proteins (65). Thus important information about the kinetics of heavy meromyosin (80), myosin V, (25) kinesin (42, 82), myosin VI (81), gizzard heavy meromyosin (2, 41, 61), myosin X (69), plant myosin XI (76) and dynein (60) have been gathered. But it is not clear how the \textit{in vitro} measurements relate to the \textit{in vivo} situation where high protein concentrations lead to exclusion effects (53) and where motor proteins encounter obstacles when actively transporting material throughout the cell (65). In the case of muscle, the situation is further complicated by high density of muscle proteins (1).

To ensure that only a few XBs contribute to the signal, we genetically produced Light Chain 1 (LC1). It was labeled at cysteine 178 with the reactive dye SeTau-maleimide and deliberately inefficiently exchanged with the

\(^1\) In principle kinetic behavior can be deduced from large number of molecules by synchronizing them by applying transients [e.g. tension transients (34)(14)]. Onsager principle [predicts that observations of the relaxation of macroscopic concentration gradients yields the same chemical rate constants as the statistically averaged time course of the fluctuations as used in mesoscopic measurements. But imposing rapid gradients disturbs steady-state and is technically difficult without introducing extra series elasticity and inhomogeneities.
Degree of order and kinetics of phosphorylated (P) and de-P cross-bridges native LC1 of myofibrils. Inefficient exchange assured that there were only a few XBs in the Detection Volume (DV). We recorded the parallel (∥) and perpendicular (⊥) components of fluorescent light emitted by the dye and calculated polarization of fluorescence (PF), the normalized ratio of the difference between these two. We show that the dye is immobilized during critical transient states of the contraction cycle, in spite of the fact that it is attached to LC1 at only one point, rather than more firm attachment at 2 points (12). PF is known to be a sensitive indicator of the orientation of the transition dipole of a fluorophore (16, 17, 32, 33, 54, 57, 66, 77). We show that PF is different in different transient states of contraction cycle and hence its fluctuations reflect transitions between different states. Autocorrelation analysis of fluctuations in the PF signal revealed the kinetics of a single fluorophore. Statistical analysis revealed the distribution of orientations of XBs during contraction. We report that phosphorylation of RLC has no statistically significant effect on the kinetics and small effect the degree of order (probability distribution of orientations) of cross-bridges of contracting rabbit psoas muscle. In particular, phosphorylation has no effect on the rate of dissociation of cross-bridges from thin filaments and on the rate of execution of the power stroke.

MATERIALS AND METHODS

Chemicals and solutions. The glycerinating solution contained 50% glycerol, 150 mM KCl, 10 mM TRIS-HCl pH 7.5, 5 mM MgCl₂, 5 mM EGTA, 5 mM ATP, 1 mM DTT, 2 mM PMSF and 0.1% β-mercaptoethanol. The rigor solution contained 50 mM KCl, 10 mM TRIS-HCl pH 7.5, 2 mM MgCl₂. EDTA-rigor had the same composition except Mg was replaced with 5 mM EDTA. Ca-rigor was the same as ordinary rigor, except it contained 0.1 mM CaCl₂. Contracting solution contained 50 mM KCl, 10 mM TRIS-HCl pH7.5, 5 mM MgCl₂, 0.1 mM CaCl₂, 5 mM ATP, 20 mM creatine phosphate and 10 units/ml of 1 mg/ml creatine kinase. The relaxing solution was the same as contracting, except Ca was replaced by 2 mM EGTA and it did not contain ATP-regenerating solution. All chemicals were from Sigma-Aldrich (St Louis, MO). Labeling dye ScTau-647-monomaleimide was purchased from SETA BioMedicals (Urbana, IL).

Preparation of myofibrils. Rabbit psoas muscle bundles were washed 3 times with ice-cold EDTA-rigor solution for 30 minutes. The purpose of this step was to wash out ATP present in the glycerinating solution without causing contraction. It was followed by an extensive wash with rigor solution (not EDTA-rigor) and homogenization by the Cole-Palmer LabGen 125 homogenizer for 10 s. After a cool down period of 30 sec, it was homogenized for a further 10 sec. The reason it was homogenized in rigor solution was to avoid foam which forms when the homogenization is carried out in EDTA-rigor.
Preparation of phosphorylated and dephosphorylated muscle. Muscle fibers were naturally de-phosphorylated after a few weeks in glycerinating solution. Fibers were phosphorylated by overnight incubation in ice in a solution containing 5 mM ATP, 12.5 mM MgCl₂, 0.1 mM CaCl₂, 5 μM calmodulin, 0.5 μM MLCK, 20 mM PO₄, 30 mM KCl. Fibers were further de-phosphorylated by overnight incubation in ice in solution containing 5 mM ATP, 2.0 mM MgCl₂, 0.1 mM CaCl₂, 1 mM EGTA, 20 mM PO₄, 30 mM KCl. Phosphorylation by incubation in glycerinating solution with phosphatase enzyme inhibitor (20 mM sodium fluoride and 20 mM phosphate (67) were not used in order to avoid complications due to the direct effect of fluoride on myosin (45, 62, 64)). Soelectric focusing gel of phosphorylated (by the addition of MLCK, lane 2 and de-phosphorylated, lane 1) myofibrillar lysate of psoas muscle stained with Pro Q showed that phosphorylated RLC band was 1.52 times stronger than de-phosphorylated band..

Isoelectric focusing: To assess the degree of phosphorylation, muscle proteins were analyzed by isoelectric focusing. Total protein concentration was analyzed by the Bradford assay after homogenizing phosphorylated and de-phosphorylated muscle in 8 M urea. For isoelectric focusing a sample was diluted to 2.5 mg/ml in a buffer (9 M urea, 130 mM DTT, 20% glycerol, 250 μl of 40% carrier ampholines pH 4-6 (BioRad), 10% Triton X-100). Denaturing isoelectric focusing gel (10 M urea, 18% acrylamide/bis-acrylamide, 20% glycerol, 10% Triton-X 100, 1 ml of 40% ampholines, 60 μl of 10% APS, and 30 μl of TEMED) was run overnight at constant 350V and fixed with 7% TCA, 50% methanol for 2 hours following procedure of Cooke et al. (56, 67). The gel was washed with double distilled water for 10 minutes and stained with Diamond Pro-Q phosphoprotein gel stain (Invitrogen) for 3 hours in the dark. It was destained with 50 mM sodium acetate, 20% acetonitrile by shaking at room temperature for at least 30 min.

Expressing LC1: Recombinant vector (pQE60) containing LC1 with a single cysteine residue (Cys178) was donated by Dr. Susan Lowey (University of Vermont). It was transfected into E.coli M15 competent cells. Recombinant clones were selected by resistance to ampicillin. That the sequencing was correct was confirmed by an independent company (Iowa State University of Science and Technology). To over-express LC1, Luria broth containing 100 μg/ml of ampicillin was used and induction was achieved with IPTG. His-tagged LC1 was affinity-purified on a Ni-NTA column.. The fractions were eluted by imidazole and were run on SDS-PAGE. This was followed by a Western blot analysis with anti-LCN1 antibodies (Abcam, CA). LC1 fractions were pooled and dialyzed against 50 mM KCl and 10 mM phosphate buffer pH 7.0. SDS-PAGE showed that the dialyzed protein exhibited a single ~25-kDa band. Protein concentration was determined using the Bradford
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Assay. In some experiments, commercial skeletal human LC1 was used (Prospec, Ness Ziona, Israel).

Choice of the dye: The fluorophore of choice must be is excited in the red to bypass most contributions by autofluorescence (37), and therefore the following two dyes were suitable choices: SeTau647 or Alexa647. SeTau was selected mainly because it was more resistant to photobleaching and brighter than Alexa647. Fig. 1 compares the photobleaching of a solution of 1 μM SeTau647 and 0.7 μM Alexa647. At these concentrations both dyes have the same extinction coefficients at the excitation wavelength used (640 nm). Further reasons to select SeTau were that is has large Stokes shift (44 nm) as compared to Alexa647 (20 nm) and high extinction coefficient and quantum yield (0.65).

The initial rate of photobleaching was 10.1 s⁻¹ for Alexa 647 and 2.4 s⁻¹ for SeTau. The diffusion of dye molecules were slowed down by the addition of 80% glycerol to increase the time they spent in the laser beam before diffusing out. Photobleaching is more dramatic in the case of muscle, in which molecules are not merely slowed down but the same XBs are illuminated for the entire experiment (20 s). The protection from photobleaching of SeTau is achieved by nano-encapsulation of the squaraine moiety of the dye chromophore system in a mixed aliphatic-aromatic macrocycle. Although SeTau has a slightly lower extinction coefficient at 640 nm than Alexa647, its overall fluorescence is more than compensated by its high quantum yield (0.65) and by the fact that its quantum yield increases 70% beyond that of Alexa647 upon protein binding. All in all, after 1 s exposure to 640 nm excitation, the fluorescence intensity of SeTau was 4.2 times larger than fluorescence intensity of Alexa647. All these attributes are necessary for extracting the maximum number of photons from a few molecules.

Labeling of LC1: LC1 (and not RLC) was chosen as the site of labeling to ensure that the phosphorylation site is not disturbed by the dye. Labeling RLC can conceivably disturb the extension of the N-terminal of RLC and phosphorylation of Ser15. It was labeled at position 178 with a 5 molar excess of protein over SeTau overnight on ice. After initial purification by dialysis against 50 mM KCL, 10 mM phosphate buffer pH 7.0, labeled LC1 was passed through a Sephadex G50 LP column to eliminate free dye. The dye and protein were complexed in a 0.2:1 ratio.

Cross-linking. Contracting myofibrils must be completely still while recording polarized fluorescence. Otherwise, fluctuations could be attributed to the “number” fluctuations, i.e. change in the number of fluorescent XBs in the detection volume as the myofibril moves through the volume. In addition, we want to
avoid muscle shortening during LC1 exchange. While the exchange solution contains ATP and EGTA, it is possible that some Troponin C becomes defective during the exchange and some myofibrils become Ca-insensitive. To completely immobilize myofibrils without affecting their ATPase, we treated myofibrils with a water-soluble cross-linker 1-ethyl-3-[3-(dimethylamino)-propyl]-carbodiimide (EDC) (3, 30, 78). 20 mM EDC was added to 1 mg/mL myofibrils in Ca-rigor solution. Myofibrils were incubated for 20 min at room temperature. The reaction was stopped by adding 20 mM DTT. The pH of the solution remained unchanged throughout the 20 min reaction. We measured ATPase by malachite green phosphate assay method, and confirmed that under our conditions X-linking had no effect on ATPase. The absence of shortening was verified by labeling the myofibrils with a 10 nM rhodamine-phalloidin and observing contraction in a TIRF microscope. No shortening was observed.

**LC1 exchange into myofibrils.** To assure small number of XBs in the DV we incubated myofibrils under very mild conditions (20 min at 30°C (79)) with very low concentration (5-10 nM) of SeTau-LC1 (the usual exchange takes place at 37°C for 1/2 hr.). 1 mg/mL of freshly prepared myofibrils were incubated with fluorescently tagged LC1 in the exchange solution (similar to the one used in (39)): 15 mM KCl, 5 mM EDTA, 5 mM DTT, 10 mM KH₂PO₄, 5 mM ATP, 1 mM Trifluoperazine (TFP), and 10 mM imidazole (pH 7).

**Data collection.** Myofibrils are inefficiently exchanged, as described above, with SeTau-LC1. The 640 nm excitation light beam is focused to the diffraction limit on the overlap band of a myofibril. Fluorescence is collected few ns after the initial pulse of exciting light is delivered. Fig. 2 explains how this procedure eliminates the contribution of scattered light: Since scattered light has no fluorescence lifetime, this eliminates contribution of light scattering from our experiment.

The laser beam is next redirected to a neighboring half-sarcomere adjusting the laser power to make sure that each sarcomere provides a similar photon rate. If this power adjustment is not done, the differences between FWHM's of PF become statistically un-interpretable (51) (also see Discussion below). The process is repeated 20-30 times, i.e. we obtain 40,000 - 60,000 PF values from each myofibril.

The Detection Volume was estimated by measuring the FWHM of an image of 20 nm fluorescent beads in the axial and lateral dimensions, which were 700 nm and 400 nm, respectively. The theoretical detection volume (DV) is equal to (π/2)^3/2 × (0.400 μm)^2 × (0.700 μm) = 0.6 μm³. Because of edge effects, the actual DV is 2.8 times greater = 1.7 μm³ (7). The concentration of myosin in muscle is 0.1 mM (1) therefore the DV contains
~3x10^5 myosin XBs. The number of observed (fluorescent) XBs in this volume is estimated to be 4 (because of the inefficient exchange) (see Results). A PicoQuant MicroTtime 200 confocal system (PicoQuant, Berlin, Germany) coupled to an Olympus IX71 microscope was used to acquire the fluorescence data as described earlier (51). Before each experiment, fluorescence of an isotropic solution of a dye with long fluorescence lifetime (50 nM rhodamine 700) was measured, after first splitting into the parallel and perpendicular channels. Since rhodamine 700 is small (and hence its solution is nearly isotropic), the signal into each detector was attenuated to make sure that they gave identical readings. The PicoQuant is the time-resolved instrument capable of lifetime imaging with Single Molecule Detection (SMD) sensitivity. Each photon is recorded individually by the Time-Correlated Single Photon Counting electronics in Time-Tagged Time-Resolved mode. A 640 nm pulsed laser provided linearly polarized excitation parallel to the myofibrillar axis, which was always vertical. Fluorescence was collected 7.36 ns after the beginning of the excitation pulse, or 8.8 ns after the arbitrary chosen start point of the measurements (see above). A 60x, 1.2 NA water immersion objective of the Olympus IX71 collected fluorescent light, and passed it through a 650 nm long pass interference filter before going through the confocal pinhole. A birefringent prism separated the vertically and horizontally polarized fluorescent light, and Avalanche Photodiodes measured the emitted light. Fig. 3 shows images of a contracting myofibril:

**Statistical analysis.** Origin v.8.6 (Northampton, MA) was used to compute autocorrelation functions and fit the data by a non-linear (Levenberg-Marquardt) algorithm for \( \chi^2 \) minimization. SigmaPlot 11 (Systat Software, San Jose, CA) was used to compute histograms.

**RESULTS**

The number of observed myosin molecules. Fluorescence Correlation Spectroscopy (FCS) analyzes the fluctuations in the signal as fluorescent molecules in solution diffuse through the detection volume. It is well established that the autocorrelation function of fluctuations at delay time 0 is equal to the inverse of the number of molecules contributing to fluctuations \( N = 1 / \text{ACF}(0) \) (19, 21, 44). Six ACF’s were obtained from solutions of the fluorophore in the range 1.29 - 77.3 nM. Extrapolating the plot of concentration vs. \( 1 / \text{ACF}(0) \) to 1 molecule, revealed that a single molecule of SeTau illuminated with 0.2 \( \mu \)W of laser power corresponded to ~75 counts per channel [i.e. total fluorescence \( I_{\text{total}} = I_\parallel + 2 \times I_\perp \) from one molecule of SeTau at 0.2 \( \mu \)W laser power was 225 counts/s (51)]. We use this fact to estimate the number of molecules contributing to the signal. Fig. 4A shows the intensities of perpendicular and parallel channels of a typical contracting myofibril. The polarized
intensities in $\parallel$ (ch3) and $\perp$ (ch2) channels were 2.45 and 1.13 counts/ms respectively giving total intensity $I_{\text{total}} = I_{\parallel} + 2 \times I_{\perp} = 4.71$ counts/ms. Therefore with the power of the laser of 0.2 μW the number of observed molecules is 20. The actual power used varied between 0.2 and 0.4 μW (to keep the count in ch1 at approx. ~2 counts/ms) so we estimate that there were between 20 and 40 XBPs in the DV. However, it should be emphasized that as long as the number of cross-bridges is mesoscopic, the exact number does not matter, i.e. 20 molecules should give the same result as 40 molecules etc.

Approximately 20 rotating cross-bridges imply that the fluctuating signal is 22% of the mean. The precision of measurement is approximately equal to $1/\sqrt{X}$, where X is the number of fluctuations detected. Therefore to get 1% precision we have to analyze 10,000 fluctuations. The characteristic lifetime of fluctuation due to rotations of the lever arm is of the order of ms (34). Therefore 10,000 fluctuations will occur in 10 s. We collected the data over 20 s.

**Kinetics**

XBPs constantly change orientation while undergoing the mechanochemical cycle, thus causing fluctuations of PF. PF fluctuate about their equilibrium values with certain rate constants. The transitions between various enzymatic and mechanical states of muscle are governed by a Poisson process. A single fluctuation cannot predict a chemical rate constant, but the rates of many fluctuations are the same as rate constants that govern macroscopic dynamic processes (58). For certain processes the effect of fluctuations on the rate constants were analytically predicted (22). Here we attempt to predict rate constants from fluctuation caused by a rotating XB. An autocorrelation function, $ACF(\tau)$, of FP fluctuations accomplishes this when fluctuations are due only to XBPs. By definition, $ACF(\tau) = <\delta FP(t)\delta FP(t+\tau)>$ where $<$ denote averaging over a long time-period (20). The system is in a steady-state (dependence of $ACF(\tau)$ on t is eliminated). Fluctuations of PF as in Fig. 3D can arise from (i) random noise; (ii) a change in the number of fluorophores in the DV (22, 44); (iii) a change of orientation of the SeTau transition dipole imbedded in LC1. The contribution (i) to fluctuations is eliminated because random noise is uncorrelated. The contribution (ii) is not significant because each myofibril is cross-linked and does not change in length, so no fluorophores can enter or leave DV during contraction. In any case, a change in signal can only be caused by rotation, and myofibrils do not twist during contraction. We conclude...
that the fluctuations arise solely because of a change in orientation of the SeTau transition dipole imbedded in LC1. The autocorrelation function is initially large because the average product of a fluctuation amplitude at some time \( t_0 \) and at short time later, \( \tau \), must be large, because the amount of fluctuation at time \( t_0 \) (\( \delta F(t_0) \)) is not much different than \( \delta F(\tau) \). The average of the product \([\delta F(t_0)\delta F(t+\tau)]\) is close to its maximal value of \((\delta F(\tau))^2\).

Since the signal average is 0, when \( \tau \) is large the amplitude is likely to be the same above and below the average, i.e. on the average \( \delta F(t_0)=-\delta F(\tau) \). Therefore the autocorrelation function at long \( \tau \) tends to decay to 0. The rate of decay of autocorrelation function from maximal value to 0 is a reflection of how quickly the average fluctuation crosses the 0 baseline.

The exact shape of the autocorrelation function depends on the model assumed for the chemical reaction that drives the cycling of the XBs. In the past we used a simple ON-OFF model, where cross-bridges were either immobilized (attached) to thin filaments or mobile (detached) from thin filaments (51). Here we use more realistic model. The model must include transitions between three fundamental states as illustrated in Fig. 5. Each state has to be characterized in terms of mobility of LC1-SeTau. This is done by measuring the time 0 anisotropy, steady-state anisotropy and the lifetime of SeTau in each of the transient states. A FluoTime 200 fluorometer (PicoQuant, Inc.) was used and measurements were done at room temperature as in (50). The sample was excited by a 635 nm pulsed (at 20 MHz) diode laser and observed through a 670 nm monochromator with an additional 650nm long pass filter. The time resolution was <10 ps and the FWHM of the pulse response function was less than 100 ps. The anisotropy decays and lifetimes were analyzed by a multi-exponential model using FluoFit software (PicoQuant, Inc.). Typical records of anisotropy decay of SeTau coupled to LC1 showed that the anisotropy decay was composed entirely of a single exponential decay.

Records of SeTau-LC1 coupled to myofibrils gave steady state anisotropy=0.071.

The cycle begins with a pre-hydrolytic state MT, in which myosin (M) has dissociated from thin filament by binding ATP (T), which is hydrolyzed to assume a post-hydrolytic MDP state (43). After hydrolysis the products remain bound to M. The MDP state is characterized by partially open cleft between upper and lower subdomains of the 50KD domain as well as a lever arm in the UP position (13). This complex, as well as MT, are free to rotate in the myofilament space, and have low steady-state anisotropy \( a_1 \) (Table 1). Next, the XB binds to actin to assume a pre-power stroke state AMD, which becomes partially immobilized. Table 1 shows
that its steady state anisotropy becomes $0.116^2$, which is represented in Fig. 5 by value of $a_2$. The AMDP state is characterized by a partially open cleft between the upper and lower subdomains of a 50KD domain and a lever arm in the UP position (13). Next, dissociation of P from AMDP state initializes a XB power stroke involving transition to a rigor-like AMD state and a final rigor (AM) state. This state has the highest steady-state anisotropy $a_3$. AMD and AM states are characterized by a closed cleft and the lever arm in the DOWN position. **Table 1** shows that the absence of a nucleotide further increases steady-state anisotropy. Transition from AMDP to AM is a power stroke. This is represented in Fig. 5 as a transition $a_2$ to $a_3$. This may occur in several steps (13, 34). Finally the XB rapidly dissociates from actin by binding fresh molecule of ATP, (assuming low anisotropy $a_1$) after which the cycle repeats itself.

<table>
<thead>
<tr>
<th>State</th>
<th>Time zero anisotropy $R_0$</th>
<th>Steady-State Anisotropy $a$</th>
<th>Correlation Time (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC1</td>
<td>0.325</td>
<td>0.077</td>
<td>0.777</td>
</tr>
<tr>
<td>AM</td>
<td>0.225</td>
<td>0.210</td>
<td>1.484</td>
</tr>
<tr>
<td>MT</td>
<td>0.232</td>
<td>0.071</td>
<td>0.780</td>
</tr>
<tr>
<td>AMDP</td>
<td>0.236</td>
<td>0.116</td>
<td>0.999</td>
</tr>
</tbody>
</table>

The table shows that the dye does not need to be attached at two points to the light chain, although the procedure described in (12) is more attractive. Of course, it needs to have a well-defined PF in different enzymatic and mechanical states of the muscle.

The autocorrelation function of such a system is (47):

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2 Notice that time zero anisotropy of AMDP complex is diminished. We do not understand why this is so. Perhaps the electronic structure of the dye has changed.
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\[ R_3(t) = \frac{(a_1k_1 k_3 + a_2k_1 k_2 + a_3k_1 k_2)^2}{(k_1 k_3 + k_2 k_1 + k_1 k_2)^2} + \]
\[ \frac{k_1 k_2 k_3 (A\kappa + B)}{2\kappa (k_2 k_3 + k_1 k_1 + k_1 k_2)^2} \exp\left(-\frac{1}{2}(k_1 + k_2 + k_3 - \kappa)t\right) + \]
\[ \frac{k_1 k_2 k_3 (A\kappa - B)}{2\kappa (k_2 k_3 + k_1 k_1 + k_1 k_2)^2} \exp\left(-\frac{1}{2}(k_1 + k_2 + k_3 + \kappa)t\right) \]

\[ \kappa = \sqrt{k_1^2 + k_2^2 + k_3^2 - 2k_2 k_3 - 2k_3 k_1 - 2k_1 k_2} \]  \hspace{1cm} (2)

\[ A = a_1^2 (k_2 + k_3) + a_2^2 (k_3 + k_1) + a_3^2 (k_1 + k_2) - 2(k_1 a_2 a_3 + k_2 a_3 a_1 + k_3 a_a_2) \]  \hspace{1cm} (3)

\[ B = a_1^2 (k_2^2 + k_3^2 - k_1 (k_2 + k_3)) + a_2^2 (k_3^2 + k_1^2 - k_2 (k_3 + k_1)) + \]
\[ + a_3^2 (k_1^2 + k_2^2 - k_3 (k_1 + k_2)) + 2(k_1 k_3 - k_1^2) a_2 a_3 + \]
\[ + 2(k_1 k_2 - k_3^2) a_1 a_2 \]

Origin 8.6 was used to fit the experimental data (circles) to the R₃. The red lines in Fig. 6 are best non-linear fit to eq. 1. Shown are 4 representative traces from 28 experiments on MLCK-phosphorylated muscle. Fluoride has strong effect on enzymatic properties of myosin (8, 45, 62, 64). To avoid using fluoride to inhibit dephosphorylation, phosphorylation was achieved by adding MLCK, as described in the METHODS.

The data could not be fitted with 2-state (ON and OFF) mode. The fit was not so good for de-phosphorylated myofibrils. The average values of a and k were determined by experiment. It remains to assign experimental k-values to the model. The average values are summarized in Table 2.

**Table 2.** Experimental values of the kinetic coefficients from 26 experiments on de-phosphorylated and phosphorylated XB's in contracting psoas myofibrils.

<table>
<thead>
<tr>
<th>Myofibrils</th>
<th>k₁(s⁻¹)</th>
<th>k₂(s⁻¹)</th>
<th>k₃(s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>De-Phosphorylated</td>
<td>961±560</td>
<td>1.9±2.7</td>
<td>0.19±0.14</td>
</tr>
<tr>
<td>MLCK Phosphorylated</td>
<td>689±484</td>
<td>0.30±0.30</td>
<td>0.18±0.20</td>
</tr>
</tbody>
</table>
The assignment of $k_1$ is obvious: it is the fastest of the processes, therefore it must be myosin head dissociation from thin filaments: $AM \rightarrow MT$. $k_2$ and $k_3$ are symmetrical in equations 1-3. However, the rate of myosin binding to actin is more likely to be higher than the rate in the power-stroke. Since the rate $k_2$ is higher (at least for de-phosphorylated muscle) than $k_3$, we assign $k_2$ as the rate of myosin head binding to actin and $k_3$ as the power-stroke rate.

None of the differences in k’s of phosphorylated and de-phosphorylated myofibrils were statistically significant, primarily because SD of measurements was so large. The difference in $k_1$ of -261.782 was not statistically significant ($t = -1.625$, $P = 0.112$ with 41 degrees of freedom). The 95 percent confidence interval for difference of means was -543.615 to 128.311. The difference in $k_2$ of -1.642 was not statistically significant at 95% confidence level. The difference in $k_3$ of -0.0122 was not statistically significant ($t = -0.223$ with 40 degrees of freedom, $P = 0.824$). The 95 percent confidence interval for difference of means was -0.123 to 0.0986.

We conclude that phosphorylation of RLC induces no obvious changes in the mechanochemical cycle of myosin. We can only say that, under ex-vivo conditions, the mean rate of XB dissociation of actin is 825 s$^{-1}$, the mean rate of myosin binding to thin filaments is 1.1 s$^{-1}$, and the mean rate of the power stroke is 0.19 s$^{-1}$.

**Distribution of XB orientations**

The easiest way to characterize spatial distributions of the transition dipole orientations of the dye is to plot polarization values vs. the number of times that a given orientation occurs during a 20-second experiment. Fig. 7 shows representative probability distributions of de-phosphorylated myofibrils.
Table 3 is a comparison of FWHM's of 28 probability distributions of phosphorylated and de-phosphorylated myofibrils.

<table>
<thead>
<tr>
<th>Myofibrils</th>
<th>FWHM</th>
<th>Mean Polarization</th>
<th>Mean Intensity (counts/ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>De-Phosphorylated</td>
<td>0.47±0.04</td>
<td>-0.13±0.03</td>
<td>2.305±0.336</td>
</tr>
<tr>
<td>MLCK Phosphorylated</td>
<td>0.50±0.03</td>
<td>-0.23±0.03</td>
<td>1.416 ±0.387</td>
</tr>
</tbody>
</table>

The difference in FWHM, while small in absolute terms (6%) was statistically significant (P = 0.012, t = 2.634 with 45 degrees of freedom). The difference of the mean polarization of -0.107 was also statistically significant (t = -10.209 with 45 degrees of freedom, P = <0.001). 95 percent confidence interval for difference of means was -0.128 to -0.0859. We conclude that phosphorylation makes a small difference to the degree of order of active cross-bridges.

DISCUSSION

We studied the effect of phosphorylation on skeletal cross-bridge kinetics and the distribution of orientations during steady-state contraction of muscle. By sacrificing the idea of measuring the signal from an individual XB and instead concentrating on detecting the signal from a few molecules, we gained the ability to increase the time resolution of ex vivo measurements in muscle to a few ms. To achieve this we exchanged fluorescent LC1 with native LC1 of myofibrils under exceedingly mild conditions (see METHODS). Such mild labeling required the use of microscope with single molecule sensitivity such as the PicoQuant MicroTime 200. In a typical experiment we observed about 20 XBs. Minimization of inhomogeniteties was possible by focusing the exciting laser light on a single half-sarcomere. The dye of choice was SeTau which was excited in the red and observed in the far red. Using SeTau dye decreased photobleaching, increased signal intensity and decreased autofluorescence. The fluorescent signal was gated, assuring no interference from scattering.

Kinetics. Determination of $k_1$, $k_2$ and $k_3$. Dissociation of the myosin head from the thin filaments is the most rapid process occurring during contraction. Since dissociation is the only rapid process detected by our method, it was natural to assign it to $k_1$. The SD of the means of phosphorylated and de-phosphorylated myofibrils was so large as to make the differences between the means statistically insignificant. The average value of $k_1$ was 825 s$^{-1}$. Similarly, the large value of the SD of the means of $k_2$ of de-phosphorylated myofibrils was so large as to make the difference statistically insignificant. The mean rate of myosin binding to thin filaments was 1.1 s$^{-1}$. 

Finally, there was no statistical difference between the rates of power strokes in phosphorylated and de-phosphorylated myofibrils $k_3$ (the average rate was $0.19 \, \text{s}^{-1}$).

The detection of a few myosin molecules in ex-vivo ventricle with millisecond time resolution is not technically easy. The main problem is that the signals are weak. Weak Gaussian signals with low Signal-to-Noise (S/N) ratio have intrinsically large relative FWHM, and this applies as well to the ratio of these signals, such as polarization of fluorescence, as shown by (51). In addition, there are at least 5 reasons for such a large FWHM or SD of measurements: #1. Non-uniform incorporation of LC1 into sarcomeres (Fig. 3); #2. Incomplete degree of phosphorylation of myofibrils (Fig. 1S); #3. Low steady-state anisotropy of SeTau and relatively small differences in anisotropy between different mechochemical steps (Table 1); #4. Large variability between samples. Ventricles are prepared in Florida and stored for various periods at -80°C. They are then shipped on ice to Texas, and stored for a few days at -20°C before experiment is done. Each of these steps imposes some variability on the quality of the sample; #5. The fitting program must estimate 6 parameters. This allows it a great freedom in selecting the best fit. Most of these reasons are beyond our control, e.g. non-uniformity of muscle labeling (#1) is caused by extremely low concentration of the dye, #3 is an intrinsic property of SeTau and replacing it with Alexa647 would result in even smaller S/N, #5 could have been reduced by enforcing limits on the "a" values but it would have imposed additional arbitrary elements on the model. More experiments are needed to decrease SD, e.g. to make more measurements at room temperature, perform additional measurements at 37°C, and better control #2 and #4.

No effect of phosphorylation of RLC is consistent with the earlier conclusion of Metzger et al. (49) that phosphorylation of RLC plays no role in the rate of tension redevelopment and maximum isometric tension at saturating concentrations of Ca$^{2+}$. It is also consistent with the earlier result from this laboratory which suggested that the duty cycle was un-affected by phosphorylation (51), suggesting that isometric force was not changed by phosphorylation of RLC. No effect on force was also observed at saturating Ca$^{2+}$ concentrations in skeletal (73) and cardiac (55) muscle, consistent with earlier results (4). The fact that the rate was so slow leads to the conclusion that prevention of shortening by cross-linking slows down, but does not does not prevent XBs from executing power-strokes, suggesting that they can reach neighboring target zones. If they were unable to reach neighboring target sites, no fluctuations due to rotation of XBs would have occurred, i.e. ACF would have been flat. The mechanical cycle goes on in spite of cross-linking, like the enzymatic cycle (29). Further, we can conclude that XBs act independently from each other, i.e. they are unsynchronized. If they were synchronized, the ACF would be periodic, because autocorrelation function of a periodic signal is periodic (5).
Overall, our kinetic results suggest that phosphorylation at saturating [Ca²⁺] has no effect on maximal isometric tension (59, 68, 71). The situation is different at low [Ca²⁺] where phosphorylation is able to increase force because the fraction of XBs in force generating state is low (6, 70). The situation is also different in muscles carrying familiar hypertrophy mutations which show obvious differences in kinetics when compared to wild type muscles (18, 27, 28, 36, 47, 48, 52) and even between α- and β-Cardiac myosins (40).

**Distribution.** Comparison of the probability distributions of orientations of XBs containing phosphorylated and de-phosphorylated RLC can be misleading unless the means of distributions are the same (51). This is because the relative FWHM of a distribution³ is sharply dependent on the strength of the signal⁴. Due to the stochastic nature of the Gaussian signal, a small mean is associated with a broad distribution (large relative FWHM) and a large mean is associated with a narrow distribution (small relative FWHM). The same is true for the ratio of two Gaussian signals (like polarization of fluorescence). Therefore if the means are different, an impression is created that the distributions are different. Meaningful comparison of FWHM’s or SD's of the ratio of two Gaussian signals requires that they have nearly equal mean. (It is not always possible to achieve exactly the same mean especially when the signal is as weak as in our case).

The absence of an effect from phosphorylation on the kinetic rate and FWHM is illustrated schematically in **Fig. 8**. The white, blue and red spheres symbolize LC1, and phosphorylated and de-phosphorylated RLC, respectively. The transition dipole of SeTau is shown as a white arrow. The violet cones represent a 3D Gaussian distribution of SeTau transition moments. The absence of an effect on kinetics is represented by the fact that the left (phosphorylated) XB is in the same transitional state (here dissociated from thin filament) as the right (de-phosphorylated) one. The small effect on FWHM is represented by the fact that the violet cone within which phosphorylated XB rotates is slightly (6%) more open.

We have previously found that **relaxed** XBs of muscle in which RLCs were de-phosphorylated were organized more tightly than XBs of muscle in which RLC's were phosphorylated (51). We also found small differences in the kinetics and orientational distribution of XBs. The differences observed in active XBs are likely due to the

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³ Since the distribution is normal, FWHM=2Sqrt(2ln2)=2.355 SD (Standard Deviation).

⁴ In practice, we ensure that the average photon counts in each series of experiments are similar by keeping the photon rate constant by adjusting the power of the illuminating laser.
fact that in earlier work muscles were phosphorylated by the addition of phosphatase inhibitors. These contain a high concentration of fluoride that may affect the kinetics and distribution of XBAs during contraction. Also, the kinetic constants were obtained from a two-state (ON-OFF) model, which may be too great of an oversimplification.

**Perspectives and Significance.** There are convincing evidence that in cardiac muscle phosphorylation enrichment increases isometric force and peak power output and both maximum shortening speed and the shortening velocity at peak power. This suggests that reduced RLC phosphorylation is a key aspect of impaired contractile function in the diseased myocardium (e.g. Ferenczi, (15, 75)). The future research on this subject must therefore concentrate on the role of phosphorylation of **cardiac RLC**. Phosphorylation of skeletal RLC is perhaps a remaining vestige of evolution of cardiac myosin. The mechanical changes observed earlier - e.g. (10), (46, 72) - are perhaps inevitable consequences of adding a negative charge to the lever arm of myosin.
ACKNOWLEDGMENTS

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REFERENCES


Degree of order and kinetics of phosphorylated (P) and de-P cross-bridges


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LEGENDS TO FIGURES

**Fig. 1.** Comparison of photobleaching of Alexa 647 (**A**) and SeTau (**B**). The photobleaching occurred in 2 phases: the rate of the first phase, lasting ~0.5 s was 10.1 s⁻¹ for Alexa. It was 4.2 times slower for SeTau. The rate of the second phase was 0.036 s⁻¹ for Alexa and ~3 times slower for SeTau. Note that the mean count rate of SeTau was 4.2 times larger than that of Alexa. The laser (640 nm) power was 0.9 mW before the objective.

**Fig. 2.** Fluorescence decay of parallel polarized light from rigor myofibril. **A:** no gate applied, i.e. the signal collection is started at the peak of the signal (violet line) and continued through the complete fluorescence decay. This includes scattered light (arrow). **B:** the residuals have significant dip at the time corresponding to scattering of light and are not well fitted by a straight horizontal line. **C:** Gating applied. In TCSPC systems, photons are collected one-by-one. Here, photons detected within 8.8 ns of the start of the measurement (an arbitrarily determined point in time with relation to the excitation pulse) are eliminated from our measurements. All that remains are photons collected 8.8 ns or later from the start of the measurement (indicated by the violet line). **D:** Residuals of the gated signal are quite flat. The same procedure is applied to perpendicular polarized light.

**Fig. 3.** Images of contracting phosphorylated myofibril, effect of gating. **A, B, E & F** are ungated images; **C, D, G & H** are the same images after gating. The color scale (in FLIM images) and contrast scale (in Intensity images) are much improved by gating. The green circle pointed to by the green arrow in **A** is a projection of the confocal aperture on the sample plane. Top row - images obtained with analyzer perpendicular to the myofibrillar axis, bottom row - images obtained with analyzer parallel to the myofibrillar axis. Notice that perpendicular images are weaker than parallel images, indicating that a sample is anisotropic. **A, C, E, G** - FLIM images; **B, D, F, H** - intensity images. The fluorescent lifetime scales are in nanoseconds, with 1.5 ns corresponding to blue and 3.4 ns to red. The intensity scales are in arbitrary units with 30 corresponding to black and 150 to white. Native myofibrillar LC1 was exchanged with 10 nM SeTau-LC1. Scale bar=5μm, sarcomere length = 2.1 μm; Sarcomere length doesn't change during contraction because of cross-linking (30, 78). Images were acquired on a PicoQuant Micro Time 200 confocal lifetime microscope. The sample was excited with a 640 nm pulsed laser and observed through a LP 650 filter.

**Fig. 4.** **A:** Typical time course of intensity of contracting MLCK phosphorylated psoas muscle myofibril. Ch3 (red) and Ch2 (black) are the fluorescence intensities polarized parallel (I||) and perpendicular (I⊥) to the
myofibrillar axis, respectively. The direction of excitation polarization is \( \parallel \) to the myofibrillar axis. The gate time was set to 0 ns. **B**: the same time course but with the gate set to 7.36 ns. Note the decrease of intensity due to elimination of light scattering. **C**: Polarization of fluorescence of ungated (blue) and gated (green) at 7.36 ns signals. To emphasize the differences, the ungated signal is plotted on top of the gated signal in **D**. Laser intensity was 0.2-0.4 μW.

**Fig. 5.** The 3 state model of muscle contraction. M - Myosin, D - ADP, P - inorganic phosphate, A - F-actin, T - ATP. The polarizations of fluorescence associated with different cross-bridge states. \( a_1, a_2 \) and \( a_3 \) are amplitude of polarizations.

**Fig. 6.** Representative traces of normalized autocorrelation functions of polarization of fluorescence of contracting MLCK phosphorylated psoas myofibril. Circles are experimental data, and a red line is the fit to equation 1. The fact that the correlation decays in time indicates that the orientation of absorption/emission dipoles change in time. The fact that ACF decays to a value >0 is due to the fact that mean polarization was non-zero (~0.23). Delay time is in seconds.

**Fig. 7.** Selected examples of the probability distributions of orientations of XBs of contracting myofibrils containing de-phosphorylated RLC. De-Phosphorylation was affected by the addition of EGTA to WT myofibrils.

**Fig. 8.** Schematic representation of the fact that phosphorylation of RLC has no effect on kinetics and minimal effect on distribution of XBs. Phosphorylated and de-phosphorylated RLC are shown as blue and red spheres, respectively. The white sphere is LC1 and white arrows are the transition dipoles of the dye. The violet cone is the cone of angles within which the transition dipole fluctuates. The thick filament is shown in dark blue, actin filament in yellow and myosin neck in green.