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Original article

# Ventricular myosin modifies in vitro step-size when phosphorylated

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ABSTRACT

Cardiac and skeletal muscle myosins have the central role in contraction transducing ATP free energy into the mechanical work of moving actin. Myosin has a motor domain containing ATP and actin binding sites and a lever-arm that undergoes rotation impelling bound actin. The lever-arm converts torque generated in the motor into the linear displacement known as step-size. The myosin lever-arm is stabilized by bound essential and regulatory light chains (ELC and RLC). RLC phosphorylation at S15 is linked to modified lever-arm mechanical characteristics contributing to myosin filament based contraction regulation and to the response of the muscle to disease. Myosin step-size was measured using a novel quantum dot (Qdot) assay that previously confirmed a 5 nm step-size for fast skeletal myosin and multiple unitary steps, most frequently 5 and 8 nm, and a rare 3 nm displacement for β cardiac myosin (βMys). S15 phosphorylation in βMys is now shown to change step-size distribution by advancing the 8 nm step frequency. After phosphorylation, the 8 nm step is the dominant myosin step-size resulting in significant gain in the average step-size. An increase in myosin step-size will increase the amount of work produced per ATPase cycle. The results indicate that RLC phosphorylation modulates work production per ATPase cycle suggesting the mechanism for contraction regulation by the myosin filament.


## 1. Introduction

Cardiac and skeletal muscle myosins are cellular movers comprised of a motor domain containing ATP and actin binding sites, a lever-arm that undergoes rotation impelling bound actin, and a tail forming the thick filament with other myosins. Muscle myosins have the central role in contraction by transducing ATP free energy into mechanical work. The lever-arm converts torque generated in the motor into linear displacement (step-size) and undergoes shear strain due to the resisting load. Bound myosin light chains, essential (ELC) and regulatory (RLC), stabilize the lever-arm [2–4]. RLC post translational modification is a recognized pathway for the thick filament regulation in cardiac and skeletal muscle [5,6]. Computational modeling indicated a role for RLC phosphorylation in cardiac contraction regulation [7]. Inheritable cardiomyopathies (ICs) are more frequently linked to myosin mutations than other sarcomeric proteins. IC mutations are located throughout the myosin molecule impacting many protein functional characteristics. Hereditary skeletal myopathies linked to myosin are less common. They lead to muscle weakness [8] or affect myosin isoforms expressed during development leading to arthrogryposis syndromes [9]. Some disease linked mutations and other normal post translational modifications affect the myosin mover specifically in its mechanical responsiveness to motor impulsive force [10]. Our long term goal is to discover how the myosin mover adapts its mechanical characteristics in response to changing demands on muscle power output or to the effects of disease. We are concerned here specifically with quantitation of myosin step-size and its adaptation by post translational modification of the RLC.

The in vitro motility assay measures actin gliding velocity over myosin immobilized on a surface. Myosin translates actin only while the two molecules are strongly bound. The myosin duty cycle is the ratio of time spent strongly actin bound divided by the time to complete the ATPase cycle. Muscle myosin has low duty cycle to enable rapid actin translation in a muscle fiber containing densely packed arrays of myosin motors [11]. Low duty cycle skeletal and cardiac myosins elude conventional single molecule assays because actomyosin dissociates quickly and the freely moving element diffuses away prohibiting further interaction. We introduced super-resolution particle tracking of Qdot-actin in the standard in vitro motility assay (Qdot assay). The in vitro motility assay has modestly more actomyosin interactions than a single molecule encounter while actin diffusion is inhibited by methylcellulose in the motility buffer. The net effect sustains the actomyosin complex while preserving a subset of encounters that do not overlap in time on a single actin filament. A single myosin step is isolated in time and space then characterized using super-resolution.

The Qdot assay performed on rabbit skeletal HMM (sHMM) indicated a ~5 nm step-size as expected for this isoform [12]. Porcine βcardiac myosin (βMys) indicated multiple unitary step-sizes of ~3, ~5, and

*Abbreviations:* βMys, β cardiac myosin (gene MYL7); cELC, Cardiac myosin ELCs (genes MYL3 or MYL4); pβMys, RLC S15 phosphorylated βMys; RLC, Cardiac myosin regulatory light chain (gene MYL2); smMLCK, Smooth muscle myosin light chain kinase.

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~8 nm with relative normalized frequencies of 12.5%, 50%, and 37.5% [13]. We proposed that the major 5 nm step is the default step identical to the unitary step in sHMM. The 8 nm step is somewhat less likely and we proposed different from the 5 nm step by involving an extra interaction with actin via the unique N-terminus of the cardiac ELC (cELC) [14,15]. The minor 3 nm step is the unlikely conversion of the 5 nm step to the full cELC bound 8 nm step. It occurs in just 1 of 8 cycles and is isolated in time from the 5 nm step by slow ADP dissociation; hence, we observe it as a separate step [16].

RLC mutants implicated in disease lower velocity, force, and shear strain [10,17] suggesting that they alter lever-arm processing of shear stress. Changes to RLC phosphorylation levels in the cardiac muscle affect power output in model organisms [5]. We investigated if RLC phosphorylation affects the unitary step-size frequency using the Qdot assay. βMys was specifically phosphorylated at S15 in RLC using a smooth muscle myosin light chain kinase (smMLCK) [1]. Phosphorylated βMys (pβMys) has 81–89% of the myosin phosphorylated. We found that phosphorylation causes a dramatic re-distribution of step-size frequencies when compared to unphosphorylated βMys providing a significant gain in average step-size for pβMys.

## 2. Materials and methods

### 2.1. Chemicals

Quantum dot 585 streptavidin conjugate (Qdot), rhodamine-phalloidin, biotin-XX-phalloidin, phalloidin, and 10% Tris–Glycine Mini Gels were obtained from Life Technologies (Grand Island, NY). Glucose oxidase was purchased from MP Biomedicals (Santa Ana, CA). Biotin free bovine serum albumin (BSA, cat # A3059) and catalase were from Sigma-Aldrich (St. Louis, MO). Other chemicals were purchased from Sigma-Aldrich or Affymetrix (Cleveland, OH). Protein concentrations were measured using the Bradford assay (Bio-Rad, Hercules, CA).

### 2.2. Protein preparations

βMys was prepared from porcine heart ventriculum as described [13,18]. G-actin was obtained from rabbit skeletal muscle acetone powder by using the method described by Pardee and Spudich [19] then stored immediately under argon gas in liquid nitrogen. Before actin was used, the frozen G-actin was thawed and spun at 160,000 ×*g* for 90 min to remove denatured actin. Biotin-XX-phalloidin plus rhodamine-phalloidin labeling of actin filaments was performed as described [13].

Phosphorylated cardiac myosin was prepared using recombinant smooth muscle myosin light chain kinase (smMLCK) as described previously with some modifications [1]. 2 μM βMys was phosphorylated with 1.2 μM smMLCK in buffer containing 30 mM KCl, 25 mM Tris–HCl pH7.4, 12 mM $MgCl_2$, 10 mM DTT, 0.1 mM PMSF, 10 μg/ml leupeptin, 5 mM ATP, 0.2 mM $CaCl_2$ and 12 μM calmodulin. After 24 hour incubation on ice, 3 mM EGTA was added to stop the reaction. ATP, smMLCK, and calmodulin were removed from the phosphorylated myosin using 2 precipitations at low ionic strength with ice cold 5 mM phosphate buffer pH 6.5. Between precipitations the phosphorylated myosin was resuspended in 0.6 M KCl and 5 mM EDTA to remove ATP. This method removed ~99% of the smMLCK and calmodulin from the phosphorylated myosin verified by SDS-PAGE.

The purified phosphorylated myosin was resuspended in 0.6 M KCl, 50 mM Tris–HCl pH 7.4, 4 mM $MgCl_2$, 2 mM DTT and 10 μg/ml leupeptin, mixed with 50% glycerol and stored at −20 °C until used. The level of RLC-phosphorylation was checked by urea gel electrophoresis stained with SYPRO Ruby and the fluorescence was quantitated with ImageJ (NIH, USA). RLC-phosphorylation level was initially 89% and decayed to 81% in ~12 h. Results were pooled from motility experiments conducted during the 12 hour interval. Unphosphorylated myosin was treated with the same steps for generating phosphorylated myosin except without the addition of smMLCK and calmodulin.

### 2.3. Actin-activated myosin ATPase

Actin-activated myosin ATPase was measured as described [4] with some modifications. Myosin stored in 50% glycerol was precipitated with addition of 12× volumes of ice cold water containing 2 mM DTT, collected by centrifugation, then resuspended in 300 mM KCl, 25 mM imidazole pH 7.4, 4 mM $MgCl_2$, 1 mM EGTA, 10 mM DTT, and 10 μg/ml leupeptin.

Actin activated ATPase measurements were done in an assay buffer containing 25 mM imidazole pH 7.4, 4 mM $MgCl_2$, 1 mM EGTA, 10 mM DTT, and 10 μg/ml leupeptin at a final concentration of 25 mM KCl. Myosin concentration was 2 μM. Actin concentrations were tested between 0.1 and 30 μM. ATPase activity was initiated by the addition of 3 mM ATP. Inorganic phosphate production was measured using the Fiske and SubbaRow method [20]. Assays were done at 21 °C.

### 2.4. In vitro motility

The βMys was prepared for in vitro motility as described [13]. In vitro motility of Qdot + rhodamine-phalloidin labeled actin was observed with through-the-objective total internal reflection fluorescence (TIRF) [21] on an Olympus IX71 inverted microscope using a 150×, 1.45 NA objective. Images were acquired with an Andor EMCCD camera ($iXon_3$ 897 with 16 × 16 μm pixels and 16 bit dynamic range) at 5 frames per second and 30 ms exposures using the software supplied by the manufacturer (SOLIS). The frame rate corresponds to 200 ms frame capture intervals indicated by Δ*t*. Intensity values were converted to photons using the conversion formula in SOLIS as appropriate for our camera and the images output in TIFF format for reading into ImageJ. Assays were done at 21 °C.

### 2.5. Super-resolution measurements

The QuickPALM software [22] identified and localized point objects that qualified for super-resolution fitting according to user specifications including minimum SNR (>25 isolating Qdots) and maximum full width at half maximum (FWHM) of 5 pixels (107 nm/pixel in object space for the 150× objective). QuickPALM analysis produced a table (SRTable) listing each qualifying particle, particle position in pixels, position standard deviation, and frame identifier. Using the SRTable, QuickPALM rendered the super-resolved particle data as single pixels per particle in the frame sequence of the original data. Rendered frames were read into ImageJ and analyzed with MTrackJ [23]. Single pixel resolution (107 nm) of the rendered images is much less than super-resolution (<10 nm). Manual tracking was needed only to link the super-resolved particle positions into a track connecting time-ordered frames. A separate program, SRTrack written in Mathematica, linked the actual super-resolved particle coordinate to the track then updated the SRTable with the frame-to-frame tracking linked list. SRTrack eliminated any incorrectly identified MTrackJ particles that did not have a super-resolved equivalent. The latter removed the effect of Qdot blinking. Representative Qdot displacement vs time data is included in Supplementary Information Fig.S1.

In any motility assay a few Qdots did not visibly move due to apparent immobilization on the glass surface. These particles were tracked at super-resolution to quantitate thermal/mechanical fluctuations.

### 2.6. Simulation

We simulated motility assay velocity event density as described previously for an actin filament 1.2 μm long [13]. Simulation generates unitary myosin binding events during successive Δ*t*'s that are converted to actin displacement by the myosin step-size then to actin velocity by