

In the present work we directly determined stiffness of individual myosin heads isolated from *M. soleus* tissue samples of affected patients using the three-bead optical trapping assay. Since each assay contained mutant and wildtype head domains, stiffness of individual heads with and without the R723G mutation could directly be compared. We determined head stiffness by imposing triangular stage displacements and recording bead displacements during binding events, taking into account compliance of the actin-bead link determined by the variance-hidden-Markov method (Smith et al., BJ 2001). We found two populations of head domains, one with a head stiffness of 0.39 ± 0.24 pN/nm the other with an about 3-fold higher stiffness. The low value is close to our previous optical trapping with β -MyHC (0.38 ± 0.06 pN/nm; Brenner et al., JMRCM 2012), the about 3-fold increase in head stiffness agrees well with our previous estimate from rigor stiffness in fibers with the same mutation (Seebomh et al., BJ 2009) while the absolute value of head stiffness from fibers was about 25% lower than determined here.

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Position of Phenylalanine in the Relay Loop is Important for Myosin Motor Action

Jinghua Ge^{1,2}, Yaroslav V. Tkachev^{1,3}, Yuri E. Nesmelov^{1,2}.

¹Department of Physics and Optical Science, University of North Carolina at Charlotte, Charlotte, NC, USA, ²Center for Biomedical Engineering and Science, University of North Carolina, Charlotte, NC, USA, ³Engelhardt Institute of Molecular Biology RAS, Moscow, Russian Federation.

We have scanned phenylalanine in the relay loop of Dictyostelium discoideum myosin to study the role of the relay loop in the myosin ATPase actin activation. It is known that C-terminus of the relay loop is on the actin binding interface. Myosin crystal structures show that F506 of the relay loop interacts with F487 of the relay helix in the post-recovery stroke structural state. This interaction is absent in the pre-recovery stroke state due to the linear shift of the relay loop along the relay helix. We hypothesized that actin binding facilitates this conformational change in the relay loop-helix region, thus activating myosin ATPase rate. The goal of the phenylalanine scan was to simulate the loop-helix shift and populate myosin pre- or post-recovery stroke structural states, mimicking proposed effect of actin binding. Two myosin mutants were designed (F506A:D505F and F506A:G507F), along with the wild type myosin (F506) placing phenylalanine in the three consecutive positions within the relay loop. We have used transient time-resolved FRET, myosin intrinsic fluorescence, pyrene labeled actin, and chemical quench to characterize myosin kinetics in detail and conclude on the role of F506 in myosin relay loop. Supported by NIH AR59621.

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Role of the Coil-Helix Transition within Loop2 in Cardiac Myosin Kinetics Modulation

Yaroslav V. Tkachev^{1,2}, Yuri E. Nesmelov².

¹Engelhardt Institute of Molecular Biology RAS, Moscow, Russian Federation, ²University of North Carolina, Charlotte, Charlotte, NC, USA.

We have used Molecular Dynamics simulations to examine structural differences of cardiac alpha and beta myosin isoforms, potentially leading to their differences in kinetics. Cardiac myosin isoforms are intensively studied due to correlation between elevated expression level of slower beta isoform and the heart failure. Relationship between kinetic properties of specific isoform and the heart function leads to the new strategy in the failing cardiac muscle treatment, direct myosin activation. Considering myosin head as the potential drug target, it is important to know how different structural elements of the molecule regulate its kinetics.

Loop2 (25k-50k loop) connects two domains in myosin heavy chain subfragment S1. Studies of chimeric proteins with interchanged Loop2 showed modulation of protein kinetics and in vitro motility. It was speculated that the major affecter of myosin kinetics is the length of the Loop 2. We have constructed models of alpha and beta cardiac S1 isoforms using available X-ray structure of beta isoform and homology modeling. Trajectories of 0.5 μ s length in explicit solvent for each isoform were calculated. Conformational analysis revealed several regions (including Loop2), adopting significantly different conformations amongst alpha and beta isoforms. The terminal regions of Loop2 in alpha myosin undergo coil-to-helix transitions. This process correlates with the breakdown of salt bridges, coupling Loop2 to the 7-stranded beta sheet in the core of myosin. In contrast, these salt bridges are conserved in the beta myosin isoform. Loop2 is connected to helices attached to P-loop and switch II loop within myosin active site. We conclude that observed coil-to-helix transition within Loop2 of alpha cardiac myosin decouples structural elements of the active site and the 7-stranded beta sheet, thus modulating kinetics of ATP hydrolysis and ADP release. Supported by NIH AR59621.

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A Kinetic Model that Explains the Transient and Steady State Responses to Mechanical and Chemical Steps Applied to Ca^{++} Activated Skinned Fibers from Skeletal Muscle

Vincenzo Lombardi, Marco Linari, Marco Caremani, Mario Dolfi.

Biology, University of Florence, Firenze, Italy.

Force and shortening in muscle are generated by ATP-driven working strokes of myosin II motors, during their cyclic interactions with the actin filament. *In vitro* and *in situ* studies suggest that the working stroke is associated with the release of phosphate (Pi). We used nanometer-microsecond mechanics on skinned muscle fibers from rabbit psoas (2.4 μ m sarcomere length, 12 °C) to record the velocity transient following a force step and found that the early rapid shortening, which represents the mechanical manifestation of the working stroke, is not affected by the increase in [Pi], while the subsequent transition to the steady shortening velocity is accelerated and the steady power at high loads is reduced. A new chemo-mechanical model has been proposed that reproduces the transient and steady state responses by assuming that biochemical and mechanical steps are not tightly coupled: (i) the release of the hydrolysis products (Pi and ADP) from the catalytic site of the myosin motor can occur at any stage of the working stroke and (ii) a myosin motor, in an intermediate state of the working stroke, can slip to a second actin monomer (the next monomer away from the center of the sarcomere) before terminating the biochemical cycle. This model explains the efficient action of muscle molecular motors working as an ensemble. Here we demonstrate the model ability to fit the force transients elicited by jumps in either [ATP] (Goldman et al., *Nature* **300**, 701-705, 1982; Dantzig et al., *J. Physiol.* **432**, 639-680, 1991) or [Pi] (Dantzig et al., *J. Physiol.* **451**, 247-278, 1992; Homsher et al., *Biophys. J.* **72**, 1780-1791, 1997). Supported by MIUR-PRIN and ECRF-2012 (Italy).

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Kinetic Characterization of Interactions between Stabilized Smooth Muscle Myosin Filaments and Actin

Brian D. Haldeman, Richard Brizendine, Josh E. Baker, Christine R. Cremona. Biochemistry, University of Nevada, Reno, Reno, NV, USA.

Using our previously described EDC-cross-linked smooth muscle myosin (SMM) filaments that are stable to ATP-induced depolymerization, we measured kinetic parameters of the SMM filament interactions with actin comparing the filamentous physiological state with monomeric forms of SMM. We use stopped-flow spectrometry, actin-activated steady state Mg-ATPase, and in vitro motility assays to measure the fundamental kinetic differences between SMM monomers and filaments. Using stopped-flow, we show that the apparent second order rate constant for ATP binding to the complex of pyrene-actin with SMM filaments is 0.21 μ M⁻¹s⁻¹, similar to HMM monomers with two heads, at 0.46 μ M⁻¹s⁻¹, and single-headed S1 at 0.47 μ M⁻¹s⁻¹. Using TIRF microscopy of filaments bound to surface-attached F-actin, we show ATP decreases the number of unphosphorylated SMM filaments bound per μ m actin, giving an apparent KATP of 4.8 μ M. We show that the ATP concentration that gives half-maximal velocities of actin moving over surface-attached phosphorylated SMM monomers is 21.2 μ M (KATP). Using inverted-geometry to measure the velocities of phosphorylated SMM filaments moving over surface-attached actin gave a much lower KATP = 8.5 μ M. Actin-activated steady-state Mg-ATPase of phosphorylated filaments gave a KATP = 9.2 μ M. The similarities in the two KATPs for filaments suggest that filament velocities and ATPase are limited by the same kinetic step, which best fits an attachment-limited over a detachment-limited model. Using TIRF, we show that phosphorylated SMM filaments move over surface-attached actin filaments until they reach the ends from which they do not detach, even upon dephosphorylation of the SMM. This behavior is specific to SMM filaments since skeletal filaments readily detach from actin filament ends. These observations suggest that phosphorylated SMM filaments that have been dephosphorylated are in a different state than unphosphorylated filaments in the presence of ATP.

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Evidence for the Presence of AM-ADP Myosin Heads in Rigor Muscle Fibers: Its Implication of the State of Myosin Heads after the End of Powerstroke

Haruo Sugi¹, Karina Hajar², Kazushige Kimura², Takakazu Kobayashi², Seiryu Sugiura³.

¹Teikyo University, Niizashi, Saitamaken, Japan, ²Shibaura Institute of Technology, Tokyo, Japan, ³University of Tokyo, Tokyo, Japan.

It is generally believed that, during muscle contraction, myosin heads (M) extending from myosin filaments bind with actin filaments (A) and perform powerstrokes associated with reaction, AM-ADP-Pi \rightarrow AM⁺Pi + ADP. The