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In vitro actin motility velocity varies linearly with the number of myosin impellers

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Abstract

Cardiac myosin is the motor powering the heart. It moves actin with 3 step-size varieties generated by torque from the myosin heavy chain lever-arm rotation under the influence of myosin essential light chain whose N-terminal extension binds actin. Proposed mechanisms adapting myosin mechanochemical characteristics on the fly sometimes involve modulation of step-size selection probability via motor strain sensitivity. Strain following the power stroke, hypothetically imposed by the finite actin detachment rate $1/t_{on}$, is shown to have no effect on unloaded velocity when multiple myosins are simultaneously strongly actin bound in an in vitro motility assay. Actin filaments slide ~ 2 native step-sizes while more than 1 myosin strongly binds actin probably ruling out an actin detachment limited model for imposing strain. It suggests that single myosin estimates for t_{on} are too large, not applicable to the ensemble situation, or both. Parallel motility data quantitation involving instantaneous particle velocities (frame velocity) and actin filament track averaged velocities (track velocity) give an estimate of the random walk step-size, δ . Comparing δ for slow and fast motility components suggests the higher speed component has cardiac myosin upshifting to longer steps. Variable step-size characteristics imply cardiac myosin maintains a velocity dynamic range not involving strain.

Keywords

Ensemble myosin strain sensing; Independent force generator; Velocity histogram probability characteristic; Random walk step-size; Actin detachment limited velocity

1. Introduction

Myosin in cardiac muscle is the molecular mover comprised of a motor or head domain containing ATP and actin binding sites, and, mechanical elements coupling motor impulsive

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force to the myosin filament backbone. Myosin mechanical coupling is through the heavy chain α -helical lever-arm domain that has two bound light chains, essential (ELC) and regulatory (RLC). The lever-arm/light-chain complex undergoes cyclical rotary movement to impel a bound actin filament. Linear actin displacement due to one lever-arm rotation is the unitary myosin step-size. In vitro actin motility velocity, v_m , is sometimes an ensemble quantity measured from single actin filaments impelled by multiple myosin motors. The myosins are immobilized on a transparent planar substrate where they hydrolyze ATP and move actin. The v_m rises linearly with cardiac muscle myosins concentration then plateaus at saturation [1]. It implies cardiac myosin stochasticity and arguably an ability to impel actin at full speed when multiple myosins are strongly actin attached. This scenario suggests velocity saturation is from myosin occupation of all available binding sites on the translating actin filament.

Alternatively, velocity saturation might imply an intrinsic dragging force from the finite time myosin spends strongly actin bound. With rising myosin concentration the overlapping strong actin binding states of many bound myosins on one actin filament will eventually halt, then reverses, the motility velocity increase due to independent actomyosin interactions. The latter describes an actin-detachment limited (ADL) barrier potentially limiting power generation in muscle contraction. How the number of actin filament bound myosins affects velocity is tested with in vitro motility. The in vitro measurement simplifies the complex structural confines of the intact muscle to just the strong actomyosin binding interactions for an ensemble of myosins, nonetheless, it directly measures velocity when 2 myosins are simultaneously bound to the same actin filament.

An ADL model is often proposed to be the principal cause of strain modulated myosin enzyme kinetics mediated by myosin light chains. The strong actin binding head in any ADL model will resist filament sliding over distances larger the average unitary cardiac myosin step-size of ~ 6 nm since any larger compliance would disable the myosin motor from moving an actin filament in the motility assay. In vitro motility assay compliance for the assay and motor used here is a known characteristic [2]. It further constrains the ADL model to resisting all measured sliding distances equal to or larger than 0.5 nm or $\sim 1/12$ of a myosin unitary step-size.

The ADL model has its mechanism embedded in each myosin while the impact is global as it inhibits movement of the whole actin filament. Single myosin mechanics indicated the strong actin binding duration for β mys, t_{on} , is exponentially distributed with a characteristic time of 14–19 ms when myosin is unloaded and saturated by ATP [3,4]. Independent force producing myosins have actin sliding velocity, $v_m = (nd)/t$, for n the number of myosins impelling actin during time t and d the unitary myosin step-size. In vitro motility experiments described here use the interval between consecutive camera frame for $t = 0.2$ s. In natural velocity units (vu) where $d/t = 1$ the ensemble actin sliding velocity, $\langle v_m \rangle = \langle n \rangle / t$ for $\langle \rangle_t$ implying averages over time t . A rational ADL model has simultaneously bound heads limiting velocity to that of a single head bound over the time that they overlap on the actin filament. In this case, in vitro velocity is nonlinear with increasing $\langle n \rangle_t$. Fig.1 indicates $\langle v_m \rangle$ vs $\langle n \rangle_t$ for the two cases of independent myosin motors (line) and when movement is inhibited by actin detachment for $t_{on} = 14$ ms (squares). For the latter, when

$\langle n \rangle_t = 10$, bound myosins overlap significantly and observed velocity is lowered by $\approx 50\%$ to an apparent $\langle v_m \rangle$ of ≈ 5.2 . Fig. 1 also shows the average number of actin bound heads over t_{on} , $\langle n \rangle_{ton}$. For $\langle v_m \rangle$ at saturation, $\langle n \rangle_{ton}$ is greater than or about equal to 1. We show here that motility velocity for ensemble β mys follows the linear relationship $\langle v_m \rangle = \langle n \rangle_t$ for $\langle n \rangle_{ton}$ up to $\beta \sim 1.8$ when we vary $\langle n \rangle_t$ by adjusting the actin filament length.

2. Methods

2.1. Protein preparation

Porcine β mys was prepared from porcine heart ventriculum as described [5,6]. Briefly, MYH7 was extracted from minced, washed ventriculum for 10 min at 4 °C with “Guba-Straub” solution (0.3 M KCl, 5 mM ATP, 1 mM DTT, 5 mM MgCl₂, 1 mM EGTA in 50 mM potassium phosphate buffer, pH 6.5). Solubilized myosin was separated from the tissue by centrifugation then three precipitation cycles eliminated contaminating soluble proteins. The pellet was then dissolved in a high salt buffer (0.6 M KCl, 5 mM ATP, 1 mM DTT, 5 mM MgCl₂, 1 mM EGTA, 0.001 mg/ml Leupeptin in 50 mM Tris-HCl pH 8.0) followed by ultracentrifugation ($250,000 \times g$ for 2 h). The upper 66% of the supernatant was collected and dialyzed overnight in storage buffer (0.6 M KCl, 2 mM DTT, 0.001 mg/ml Leupeptin in 50 mM Tris-HCl pH 7.4) followed by ultracentrifugation ($250,000 \times g$ for 3 h) to remove remaining actin or actomyosin impurities. The β mys was stored in sealed tubes at -20 °C in 50% glycerol (vol/vol). Purified β mys has less than 5% actin impurity judged from analyzing the SYPRO Ruby stained SDS/PAGE gel shown in Fig. 2. Urea gel electrophoresis and mass spectrometry analysis of the β mys did not detect any phosphorylated RLC in the Pmys used in experiments [7]. The urea gel is shown in Fig. 2.

G-actin was obtained from rabbit skeletal muscle acetone powder as described [8] then stored immediately under argon gas in liquid nitrogen. The frozen G-actin was thawed and spun at $244,000 \times g$ for 90 min to remove denatured actin before use. Rhodamine labeling of actin filaments was performed with rhodamine-phalloidin and actin in a 1.2:1 M ratio in a buffer containing 25 mM KCl, 0.2 mM CaCl₂, 2 mM MgCl₂, 10 mM DTT, 0.1 mM PMSF and 25 mM imidazole at pH 7.4 on ice for 12 h and then stored on ice until used. Just before the myosin sample was infused into the flow cell in the motility assay, 1 μ M of the rhodamine-phalloidin labeled actin was incubated with gelsolin in various molar ratios with 10 μ g/ml of leupeptin for 30 min at room temperature.

2.2. Actin-activated myosin ATPase

Actin-activated myosin ATPase was measured as described [1]. Briefly, β mys stored in 50% glycerol was precipitated with addition of 12 $\times$ volumes of ice cold water containing 1 mM DTT, collected by centrifugation, then resuspended in 300 mM KCl, 25 mM imidazole pH 7.4, 4 mM MgCl₂, 1 mM EGTA, 10 mM DTT, and 10 μ g/ml leupeptin (M-buffer). Myosin at a final concentration of 1 μ M was titrated with 0.1, 2, 4, 8, 16, 40 μ M actin. The ATPase assay buffer contained 25 mM imidazole pH 7.4, 4 mM MgCl₂, 1 mM EGTA, 10 mM DTT, 10 μ g/mL leupeptin, and a final KCl concentration of 25 mM. ATPase reaction was initiated by the addition of 3 mM ATP, and incubated at 21 °C for 5 min. Inorganic phosphate production was measured using the Fiske and Subbarow method [9]. Michaelis-Menten

fitting constants $V_{\max} = 1.5 \pm 0.13 \text{ s}^{-1}$ and $K_m = 6 \pm 2 \text{ }\mu\text{M}$ for the native βmys samples were derived using $\text{ATPase} = V_{\max} [\text{Actin}] / (K_m + [\text{Actin}])$. Errors are standard deviation for 4 measurements.

2.3. In vitro motility

βmys is adsorbed to the planar glass substrate in contact with an aqueous phase containing fluorescent tagged filament actin as described [5]. Briefly, βmys in 50% glycerol is resuspended as described above. The myosin was mixed with 1 mM ATP and 1.1 μM actin in M-buffer and centrifuged ($244,000 \times g$ for 30 min) to remove dead-heads. Myosin concentration was determined using the Bradford assay then the sample was diluted to the desired concentration. 10 μL of the myosin sample was infused into a flow cell made from the nitrocellulose coated glass coverslip and incubated for 1 min. After incubation, the flow cell was washed with 20 μL of 1 mg/ml BSA solution (BSA in M-buffer), incubated with 10 μL of 25 mg/ml BSA solution for 2 min, and then washed with 60 μL of low-salt-buffer (25 mM KCl, 1 mM EGTA, 5 mM MgCl_2 , 10 mM DTT, and 10 $\mu\text{g}/\text{mL}$ leupeptin in 25 mM imidazole pH7.4). After 1 min incubation, we did a second dead-head removal by, 2 min incubation with 20 μL of 1 μM phalloidin labeled actin in low-salt-buffer, 1 min incubation with 20 μL of 1 mM ATP in low-salt-buffer, and 1 min incubation with 20 μL of low-salt-buffer. After that, 20 μL rhodamine-phalloidin labeled actin in low-salt-buffer was infused for a 1 min incubation. Then the flow cell was washed with 20 μL of low-salt-buffer and mounted on the micro-scope stage. Once the sample was in focus, the cardiac motility buffer (25 mM KCl, 25 mM imidazole pH7.4, 4 mM MgCl_2 , 1 mM EGTA, 20 mM DTT, 10 $\mu\text{g}/\text{mL}$ leupeptin, 0.7% methylcellulose, 2 mM ATP, 3 mg/ml glucose, 0.018 mg/ml catalase, and 0.1 mg/ml glucose oxidase) was infused to initiate motility.

Fluorescence detected from the probe modified actin in the evanescent field from total internal reflection (TIR) illumination provides high contrast and time-resolved images of the actin filament movement over myosin that characterizes in vitro myosin function [10,11]. All motility velocities were measured with surfaces coated from 0.08 μM βmys bulk concentration. Moving filament fractions were >90% under all conditions. Actin sliding velocities and length of actin filaments were obtained using FIESTA software [12]. Images were collected with 100 ms exposures at $t = 200 \text{ ms}$ intervals.

2.4. Assay compliance

We previously evaluated assay compliance by assessing βmys step-size under load imposed by α -actinin [2]. There the fraction of moving actin filaments vs α -actinin concentration in a βmys motility assay indicated a linear decline for increasing α -actinin except at the highest concentrations where it was unable to fully inhibit actin filament sliding. The latter implies α -actinin is a viscoelastic inhibitor of the βmys induced actin movement [13] and that force developed by βmys during a single α -actinin/actin bound kinetic lifetime is less than the rupture force for the bond [14]. Hence compliance in the system, from intrinsic myosin elasticity [15] and myosin linkage to the nitrocellulose coated substrate, manifests as a difference in measured step-size between unloaded and loaded actin. No change in measured step-size implies compliance in the myosin linkage is negligible while compliance due to intrinsic myosin elasticity is undetectable because the α -actinin generally kinetically

detaches from actin during the course of the myosin power stroke. We found no significant change in the step-sizes due to the loading of β mys implying negligible assay compliance [2]. Experimental conditions in this previous work were identical to those used here. These results constrain expectations from an ADL model. The strongly actin bound myosin head in any ADL model must resist filament sliding over distances equal to or larger than the cardiac myosin step-size measurement error of ~ 0.5 nm since larger distances cannot be accommodated (and neglected) within assay compliance.

2.5. Velocity distributions

Filament track velocity (TV) is the average of frame-by-frame filament velocities over an entire track. Filament frame velocity (FV) exhibits frame-by-frame velocities without averaging. TV and FV histograms are corrected for contributions from thermal/mechanical fluctuations by baseline subtraction of velocities collected from filaments without net displacement. They are quantitated in natural velocity units (vu) where $\langle d \rangle / \Delta t = 1$ and average unitary D myosin step-size $\langle d \rangle = 6$ nm observed for β mys in the single myosin Qdot assay [11]. Velocity expressed in vu's allows a visual estimate of average apparent actomyosin interactions per sampling interval. The choice for $\langle d \rangle = 6$ nm is informative but arbitrary and does not impact conclusions drawn below.

Fig. 3a shows the TV and FV histograms for the 0.6 μ m long actin filament. TV and FV histograms substantially differ from each other with the TV histogram distribution featuring short trailing (low velocity) and somewhat longer leading (high velocity) tails due to the averaging over the actin track. The FV histogram distribution features a distinctively long tail at the high velocity end shown in close up in Fig. 3b. We gain a quantitative assessment of distribution shape using random variates generated from several continuous distributions that linearly relate their mean value to the mean bound myosins given by the number of actin binding sites (N) times the probability for myosin binding actin at one of these sites (p). We include Normal, Rayleigh, and Gamma distributions combined linearly to obtain reasonable fits represented in the figure as solid red (TV) and blue (FV) lines. This analysis (Mathematica Find Distribution, Wolfram Research, Champaign, IL) indicates mixtures of 2 Normal distributions (for TV) and 2 Rayleigh or 2 Gamma distributions (for FV) give reasonable estimates. The figure shows the Normal and Rayleigh Distributions fitting the data.

A Normal distribution under certain conditions is equivalent to the discrete Binomial distribution, $B(N,p)$, of velocities for parameters N and p defined above. These characteristics suggest the TV's represent principally the random binding and dissociation of the myosin impelling actin for average number bound summed over Δt , $\langle n \rangle_{\Delta t} = Np$.

The Rayleigh distribution, $R(\sigma')$, of velocities have parameter $\sqrt{\frac{\pi}{2}}\sigma' = Np'$ [16,17]. The prime ($'$) symbol denotes a binding probability possibly different from binding probability p in the Binomial distributions for TVs. The FV's represent principally the randomized displacement amplitude of the actin filament as myosin heads bind and dissociate for average number bound summed over Δt , $\langle n \rangle_{\Delta t} = Np'$. The Rayleigh distribution notably describes a random walk in 2 dimensions with parameter (in length units) $\sigma^2 = 2 D \Delta t$ for D

the two dimensional diffusion constant $\approx (\langle n \rangle_{\tau} \delta)^2 / \tau$ for random walk step-size δ , time per step $\tau = 1/V_{\text{Max}}$, and $\langle n \rangle_{\tau}$ the dt average number of actin bound myosins during τ . The Rayleigh distribution fitting the velocity histogram represents distance divided by τ measured in vu. Unifying units implies $\sigma / \tau = \sigma' (\langle d \rangle / \tau)$ giving,

$$\frac{\delta}{\langle d \rangle} = \frac{\frac{\sigma'}{\langle n \rangle_{\tau}}}{\sqrt{2V_{\text{Max}} \Delta t}} \quad (1)$$

Substituting for $\langle n \rangle_{\tau}$ in eq. (1) using $2\langle n \rangle_{\tau} = 2 \left(\frac{\tau}{t} \right) \langle n \rangle_t = \left(\frac{\tau}{t} \right) N(p + p')$ thus combining (by averaging) constraints derived from both TV and FV distributions we find,

$$\frac{\delta}{\langle d \rangle} = \frac{\sqrt{\frac{2}{\pi}} \frac{2p'}{(p+p')}}{\sqrt{2V_{\text{Max}} \Delta t}} \quad (2)$$

Eq. (2) expresses the potential divergence of TV vs FV fits. We expect $\delta \approx \langle d \rangle$ implying p/p' is constant. Comparing δ 's from 2 components of the velocity distributions indicates the dependence of the random walk step-size on the motility velocity.

The Gamma distribution, $G(\alpha, \beta)$, for α the scale and β the rate parameter have parameter $\alpha\beta = Np'$ suggesting we try $\alpha = N^{1/2}$ and $\beta = N^{1/2} p'$. The Gamma distribution does not have the familiar physical application like Rayleigh, nonetheless, it is practically useful for fitting FV distributions and an alternative to the Rayleigh distribution.

We will also use the relationship,

$$\langle v_m \rangle = \frac{1}{2} N(p + p') \quad (3)$$

for computing the average motility velocity for each motility component based on parameters measured from both TV and FV distributions. The linear or non-linear relationship of $\langle v_m \rangle$ vs N implies the same for $\langle v_m \rangle$ vs $\langle n \rangle_t$.

2.6. Velocity heterogeneity

TV and FV distributions in Fig. 3 are two component fits of the data with slow and fast components contributing major and minor areas under the curves. Multiple motility velocity components reflect apparent heterogeneity in N or p .

Parameter N is proportional to actin filament length. Actin distributions for 0.5, 0.55, and 0.6 μm average filament lengths were stabilized using 0.5, 0.25, and 0.167 gelsolin:actin ratios [18]. Actin lengths of 1.0 and 2.0 μm did not require stabilization because the standard actin preparation produced a broad distribution of lengths that were selectively sampled. All

actin filament lengths were quantitated by direct measurement from their fluorescence image. Filament lengths were approximately normally distributed with a distribution width of ~15 nm.

The actin length is tightly controlled implying likewise for N hence myosin binding probability p is the heterogeneous parameter. Probability p is proportional to myosin surface density such that the moving actin filament encounters heterogeneous myosin density. These concepts were explored previously in the context of the Qdot assay characterization of single actomyosin interactions [2].

The choice of 2 fitting components is somewhat arbitrary. We show below that the 2 component fit indicated in Fig. 3 demonstrates velocity linearity with the number of myosins bound to actin for each component. Two fitting components were also discovered independently in the Mathematica routines. We find that one component fitting does not capture the density in-homogeneity at the high velocity end suggesting the choice of 2 components appropriately balances model complexity with fitting accuracy. This concept is rigorously developed with Bayesian methods and implies the two component fit is preferable [19].

2.7. Curve fitting for distribution parameter estimates

TV and FV histograms were fitted with Binomial/Normal and Rayleigh or Binomial/Normal and Gamma distributions. The Normal distribution is used for curve fitting because it is a continuous function of velocity. Either choice (Normal and Rayleigh or Normal and Gamma) accurately represents actin motility for the β mys bulk concentration of 0.08 μ M and for each actin filament length tested with fast and slow velocity components. TV and FV histograms were fitted for each actin length with N binding sites available to the surface bound myosin, and p or p' the fitted unknown non-linear parameters. Probabilities p_f' , p_f , and p_s , p_s' for fast (subscript f), slow (subscript s) components in TV (unprimed) and FV (primed) histograms were exhaustively searched over 0–1 domains with resolution 0.04. Curve amplitudes describing fast and slow component fractions are the unknown linear parameters solved using either the generalized linear model (GLM) assuming they are normally distributed with identity link function or linear programming with amplitudes constrained to be larger than or equal to 0. Best fitting curves minimized their χ^2 difference with data and show the results from GLM.

Standard deviation estimates for non-linear parameters p_f , p_s , p_f' or p_s' were constructed by using ~100 values for their best fitting estimates. Fitting generates 4×10^5 total trial choices for the 4 free parameters at each actin filament length. Histograms for the p_f , p_s , p_f' , and p_s' choices were approximately normally distributed and fitted with a Gaussian to estimate average and variance. Average and variance estimates are essentially unchanged by using the 20–100 best parameters suggesting we are sampling a representative pool of best parameter estimates. Average and variance estimates for p_f , p_s , p_f' , and p_s' are converted to δ or $\langle v_m \rangle$ average and variance using eq. (2) or 3 with $V_{\max} = 1.5 \text{ s}^{-1}$, $\langle d \rangle = 6 \text{ nm}$ and N estimated by 15, 17, 18, 30, and 61 for the 0.5, 0.55, 0.6, 1.0, and 2.0 μ m long actin filaments, respectively, assuming 33 nm between myosin binding sites on actin filaments that are accessible to the myosin on the surface. The 33 nm repeat better approximates the motility data suggesting

the actin filament accommodates more myosins than that expected from the 35–38 nm registration length [20].

Fig. 4 show the TV (**Panel a**) and FV (**Panel b**) histograms with a fast (blue) component indicating ~14–16 vu average velocity implying ~14–16 actin bound myosins impelling the actin during t . The 15 myosin impellers place the fast component deeply in the region where the generic ADL model limits velocity (Fig. 1).

3. Results

3.1. Motility velocity histograms at various actin lengths

Fig. 5 **panels a-e** indicate TV and FV histograms for the 0.5, 0.55, 0.6, 1.0, and 2.0 mm long actin filaments. Solid lines indicate the 2 component fit using Binomial/Normal or Rayleigh distributions for TV or FV curves. Probabilities p_f , p_s , p'_f , and p'_s are estimated from fitting the TV (p_f and p_s) and FV (p'_f and p'_s) data and for fast (subscripted f) or slow (subscripted s) components as described in Methods. The broken vertical line indicates the same motility velocity in each panel and shows the incremental increase in distributed velocities with actin filament length.

3.2. Fast and slow motility components

Fast and slow motility components are minor and major contributors to the velocity histograms corresponding to 20–40 and 80–60% of the density. Fig. 4 indicates the velocity histogram (black dots) for the 1 μ m long actin filament with individual slow (solid red) and fast (solid blue) components identified. **Panel a** contains TV and **panel b** FV histograms. Fitted curves are Binomial/Normal (TV) and Rayleigh (FV) distributions. The black solid curve is the summed components fitting the data. In this case the fast component corresponds to 21% of the density.

3.3. Motility velocity vs actin filaments length

Fig. 6 **panels a & c** show motility velocity $\langle v_m \rangle$ in natural velocity units (vu where 1 vu = $\langle d \rangle / t$ for $\langle d \rangle = 6$ nm and $t = 0.2$ s) versus actin filaments length. Probabilities p_f , p_s , p'_f , and p'_s and myosin binding sites on actin, N , are used in eq. (3) to compute $\langle v_m \rangle$. Error bars indicate standard error from 15 (160,986), 6 (115,550), 20 (345,239), 5 (65,441), and 15 (32,949) assay parallels involving total number of tracking coordinates indicated in parenthesis and actin lengths running from low to high values. Black, blue, and red solid curves are best fitting for total, fast, and slow velocity components. The linear model represents the data well over a 4 fold change in the number of myosin binding sites. **Panels a & c** show results using Rayleigh & Gamma distributions for FV data fitting, respectively. The TV data is always fitted using the Binomial/Normal distribution. Lines representing $\langle v_m \rangle$ vs actin length intercepts the y-axis at 6–8 myosins implying the minimum average number needed to move an actin filament.

Comparing Fig. 1 **panel a** with Fig. 6 **panels a & c** indicates that the ADL model is very low in the observed velocity range. The broken black line in Fig. 6 **panels a & c** indicates

the ADL model estimated from $t_{on} = 14$ ms when $\langle v_m \rangle$'s converge at the y-intercept for the fast component. Observed velocities are not limited by an ADL model of the kind simulated.

We computed the myosin random walk step-size ratio using eq. (2) for fast (δ_f) divided by slow (δ_s) random walk step-sizes from the components of the myosin sliding velocity using the Rayleigh distribution for FV fitting. The ratio indicated in Fig. 6 **panels b & d** show that increasing velocity corresponds to a longer average myosin step-size.

4. Discussion

Ensemble myosin in striated muscle maintains a force-velocity curve with maximum velocity at zero force indicating when movement could be inhibited only by the internal resistance to sliding. The finite time for a strong actomyosin crosslinking during the force production step in the contraction cycle, t_{on} , could cause the strongly bound myosin to feel strain from filament displacement due to other bound motors in the ensemble impelling actin. Strain sensing in the motor is thought to be an important mechanism for mechanical regulation of heart muscle contraction [21,22]. The strain imposed by filament sliding during t_{on} is expected to be manifest in the in vitro motility assay by nonlinearity in $\langle v_m \rangle$ vs $\langle n \rangle$ as indicated in Fig. 1 **panel a**.

Fig. 6 **panels a & c** indicate motility velocity varies linearly with actin length and at significantly larger slope than that expected for motility velocity tightly coupled to t_{on} . Fast average actin displacement for the 2 μ m long filament (at ~25 vu) translates ~1.8 native step-sizes during t_{on} . Equivalently, average myosin occupation of the actin filament during t_{on} surpasses 1 when motility velocity reaches ~14 vu implying cardiac myosins behave like independent motors when simultaneously actin bound. The results rule out an actin detachment limited model for imposing strain and suggest single myosin estimates for t_{on} are too large or not applicable to the ensemble situation. We suggest that both are true with a strain sensing mechanism in myosin that distinguishes resistive load, like that from α -actinin inhibiting actin filament displacement in the motility assay [14], from accelerative load, from a second strongly actin bound myosin impelling actin in the ensemble. The former delays detachment of a strongly bound myosin cross-link effectively lengthening t_{on} while the latter rapidly detaches a strongly bound myosin cross-link effectively shortening t_{on} . Accelerative loading defined in this way can never happen when a single myosin molecule moves actin.

The TV and FV forms of the in vitro motility data are different quantitation schemes that more fully constrain motility models. These parallel quantitation methods uniquely reveal the random walk step-size for different motility speeds. Our findings in Fig. 6 **panels b & d** suggest that myosin in the fast motility component is upshifting to a longer average step-size. Complementary results were obtained in preliminary data from in vivo single cardiac myosins in beating zebrafish embryo heart ventricles. There myosin downshifted to shorter average step-size when the muscle transitioned from auxotonic to isometric contraction. We propose that average step-size adaptation mechanisms, manifesting as upshifting under accelerative load and down shifting under resistive load, permits the cardiac myosin to maintain a large velocity dynamic range without altering its ATPase kinetics.

Gordon et al. showed that force generation in a frog muscle fiber decayed linearly with decreasing overlap of thick and thin filaments and concluded that myosins in the fiber are independent force-generators [23]. Our result concerns displacement in a much different and simpler contractile system, nonetheless, the conclusion is the same within an upper limit of ~ 2 simultaneously strongly bound myosins.

Modern interests in strain-dependent biochemistry elicits comment on the ADL concept in single myosin mechanical studies as a reason to probe ADP release rates for myosin under tension [3,4,13]. It is widely accepted that t_{on} is fixed, finite, and impacting the relative sliding rate for myosin and actin filaments in a muscle. The manuscript describes the unexpected finding that a finite t_{on} has no detectable impact on sliding rate in the in vitro motility model system even when 2 myosin heads are strongly actin bound.

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Fig. 1.

Panel a. Average number of strongly bound myosins moving actin during imaging interval t , $\langle n \rangle_t$, with average velocity, $\langle v_m \rangle$, for velocity expressed in natural units (vu where $1 vu = \langle d \rangle / t$ for $\langle d \rangle = 6 \text{ nm}$ and $t = 0.2 \text{ s}$). Linearly related $\langle v_m \rangle$ vs $\langle n \rangle_t$ (line with slope 1) implies independent myosin motors. Nonlinearity in $\langle v_m \rangle$ vs $\langle n \rangle_t$ (squares) has velocity inhibition from an ADL model resisting filament sliding when 2 or more myosins are simultaneously actin bound. **Panel b.** Average number of strongly bound myosins moving actin during t_{on} , $\langle n \rangle_{ton} = (t_{on}/t) \times \langle n \rangle_t$ (line) or from simulation (squares). When $\langle n \rangle_t = 25$, $\langle n \rangle_{ton} = 1.8$ indicating conditions when there are usually (>50% of the time) 2 or more strongly actin bound myosins.

Fig. 2. SYPRO Ruby stained SDS-PAGE gel for β mys (left) and the urea PAGE gel of phosphorylated (P) and unphosphorylated (U) β mys (right). Protein bands were stained with SYPRO Ruby and the fluorescence quantitated with ImageJ. Lane 1 contains molecular weight standards for the β mys in lane 2. MHC indicates the myosin heavy chain, ELC the essential light chain, and RLC the regulatory light chain. The unphosphorylated sample (U) in Lane 3 has a faint band below the main RLC band. It is an unphosphorylated RLC with altered charge due to methylation or other post translational modification [7]. Phosphorylated RLC sample (P) in Lane 4 migrates further into the gel (pRLC) due to its additional charge. Phosphorylated β mys was prepared using recombinant smooth muscle myosin light chain kinase (smMLCK) as described [7]. Motility experiments were performed only on unphosphorylated β mys.

Fig. 3.

TV and FV histograms (red open squares and blue open circles) for the 0.6 μm long actin filament. Solid lines indicate fitted curves using 2 summed Normal distributions (red) or 2 summed Rayleigh distributions (blue). The x-axis has natural velocity units (vu where $1 \text{ vu} = \langle d \rangle / \Delta t$ for $\langle d \rangle = 6 \text{ nm}$ and $\Delta t = 0.2 \text{ s}$) and conventional units in μm/s. **Panel a** shows the full y-axis and **panel b** the expanded scale to visualize difference between TV and FV histograms at high velocity.

Fig. 4.

TV (**panel a**) and FV (**panel b**) velocity histograms for 1.0 μm long actin filaments sliding over βmys . The fast or slow component of the two component fits are indicated in blue or red. Summed distributions are indicated in black. TV and FV histograms are fitted with two component Normal and two component Rayleigh distributions, respectively. Velocity units are the same as in Fig. 3.

Fig. 5. TV (red) and FV (blue) velocity histograms for rhodamine labeled actin filaments sliding over β mys adsorbed to the substrate from 0.08 μ M bulk concentration. Actin filament lengths are 0.5, 0.55, 0.6, 1.0, and 2.0 μ m for panels a–e. Velocity units are the same as in Fig. 3.

Fig. 6.

Panel a. In vitro motility velocity, $\langle v_m \rangle$, vs actin length in μm or myosin binding sites N for cardiac myosin motility (solid lines). Black, blue, and red solid curves correspond to total, fast, and slow velocity components computed using eq. (3) with binding probabilities estimated from data in Fig. 5 and using Binomial/Normal and Rayleigh distributions as described in Methods. The velocity for myosin induced actin movement for the ADL model described in the text with $t_{\text{on}} = 14$ ms (black broken lines) is also indicated. **Panel b.** The ratio of random walk step-sizes for fast (δ_f) and slow (δ_s) components of the myosin motility velocity vs actin sliding velocity in natural velocity units (vu where $1 \text{ vu} = \langle d \rangle / t$ for $\langle d \rangle = 6$ nm and $t = 0.2$ s). Random walk step-size is computed using eq. (2). Average step-size $\langle d \rangle$ for the fast component is indicated on right vertical axis. **Panels c & d.** Same as in **panels a & b** except using Binomial/Normal and Gamma distributions to fit the experimental data as described in Methods. Error bars in all panels are standard error from 15, 6, 20, 5, and 15 assay parallels for 0.5, 0.55, 0.6, 1.0, and 2.0 μm long actin filaments.