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Structural basis for myopathic defects engendered by alterations in the myosin rod

Anthony Cammarato^{a,1}, Xiaochuan (Edward) Li^b, Mary C. Reedy^c, Chi F. Lee^a, William Lehman^b, and Sanford I. Bernstein^{a,*}

^a5500 Campanile Drive, Dept of Biology and the Molecular Biology Institute, San Diego State University, San Diego, CA, 92182, USA

^b72 East Concord Street, Dept of Physiology and Biophysics, Boston University School of Medicine, Boston, MA, 02118, USA

^c458 Sands Bldg, Dept of Cell Biology, Duke University Medical Center, Durham, NC, 27710, USA

Abstract

While mutations in the myosin S1 motor domain can directly disrupt the generation and transmission of force along myofibrils and lead to myopathy, the mechanism whereby mutations in the myosin rod influence mechanical function is less clear. Here, we used a combination of various imaging techniques and molecular dynamics simulations to test the hypothesis that perturbations in the myosin rod can disturb normal sarcomeric uniformity and, like motor domain lesions, would influence force production and propagation. We show that disrupting the rod can alter its nanomechanical properties and, *in vivo*, can drive asymmetric myofilament and sarcomere formation. Our imaging results indicate that myosin rod mutations likely disrupt production and/or propagation of contractile force. This provides a unifying theory where common pathological cascades accompany both myosin motor and specific rod domain mutations. Finally we suggest that sarcomeric inhomogeneities, caused by asymmetric thick filaments, could be a useful index of myopathic dysfunction.

Keywords

Electron microscopy; molecular dynamics; myopathy; myosin

Introduction

Muscle contraction results from repetitive cyclical interactions of myosin-containing thick filaments with actin-containing thin filaments. These filamentous systems are assembled into highly organized individual sarcomeric contractile units. Myosin, the molecular motor of muscle, contains two N-terminal “S1” globular heads followed by a long α -helical coiled-

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*Corresponding author: sbernstein@sunstroke.sdsu.edu, 5500 Campanile Drive, Dept of Biology and the Molecular Biology Institute, San Diego State University, San Diego, CA, 92182. (619) 594-5629, fax (619) 594-5676.

¹Present address: 720 Rutland Avenue, Ross 958, Division of Cardiology, Department of Medicine, Johns Hopkins University, Baltimore, MD 21205, USA.

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coil rod domain. The S1 heads hydrolyze ATP and generate contractile force while the rod-like tails are essential for associating with auxiliary proteins and for the precise self-assembly and packing of myosin molecules into filaments. Much effort has been directed at understanding the role played by S1 in the contractile process and how myosin head mutations lead to skeletal and cardiac myopathies. Less attention however has been paid to the critical roles played by the rod in these processes.

Generation of force by the sarcomere and its transmission to the extracellular matrix are often perturbed in muscle disease¹. Myopathy-associated mutations in S1 can influence myosin's chemomechanical cross-bridge cycle with actin, directly affect sarcomeric force production, alter the contractile properties of myofibers and lead to disease²⁻⁷. In contrast, the pathogenic mechanisms associated with mutations in the rod are less clearly delineated. In fact, mutations in myosin rods have been proposed to lead to distinct pathogenic sequelae relative to those in the head⁸. Here, to probe the mechanistic basis for myopathic defects engendered by alterations in myosin's rod we tested the hypothesis that disturbances in the rod, like S1 mutations, can influence myofibrillar force production and propagation, but do so by disrupting sarcomeric uniformity. We investigated the nanomechanical effects of alterations to the rod, how these perturbations influence *in vivo* sarcomeric symmetry and ultimately how they underlie the molecular basis of disease.

Results and Discussion

The S2/LMM hinge region is a relatively flexible section of the myosin rod⁹ that is critical for determining muscle-specific function^{10,11} and when mutated leads to myopathy¹². It begins near the 44 nm skip bend and is roughly localized within the second quarter and predominantly within the central third of the tail (Figure 1a). Transgenic replacement of ~25% of fast muscle myosin hinge-A in *Drosophila* indirect flight muscle (IFM) with slow muscle hinge-B (Figure 1a) increases rod coiled-coil propensity, rod and sarcomere lengths and decreases flight performance¹⁰. To characterize an additional index of myosin rod disruption due to S2/LMM hinge misexpression we compared the nanomechanical properties of the hinges and their flanking rod regions. We determined persistence length (*PL*) differences along independent segments of isolated *Drosophila* myosin molecules directly from electron micrographs (Figure 1b,c). *PL*, viz. the distance along a rod-like structure over which the tangential deflection at one end no longer correlates with a corresponding tangential deflection at the other end, frequently is used to measure the rigidity of rod-like molecules. Consistent with an increased coiled-coil propensity¹⁰ we determined higher *PL*s along discrete portions of hinge-B molecules vs. hinge-A molecules (Figure 1c). We performed 2 segmental-based analyses for roughly each one-third or each one-quarter of hinge-A and hinge-B tails. The expression of hinge-B increased *PL* along all but one segment analyzed. While a slight decrease in *PL* was determined only for the 1st quarter of hinge-B containing tails, increases in *PL* were observed for all remaining segments of hinge-B relative to hinge-A rods. Intriguingly, both independent segmental-based assessments suggest the amino acid differences initiate nanomechanical changes immediately in their region of expression. Moreover, these alterations are potentially propagated along the tail in a cumulative fashion such that the largest disparity in *PL* of the rod types occurs downstream of the hinge region in the second, one-third or the third, one-quarter segments. These differences in stiffness reflect substantial myosin rod modification, which may alter its molecular function. Specifically they suggest that distinct hinge amino acids alter critical mechanical properties of the S2/LMM hinge, influence the long-range material properties of the tail and that hinge-B rods are less flexible than hinge-A rods. In turn, alterations in the physical and as previously shown in chemical and structural characteristics¹⁰ *in vivo* could influence the assembly of myosin into uniform thick filaments.

Electron microscopy (EM) of ultrathin sections of myofibrils from hinge-B- containing transgenic IFM, reveals the presence of ultra-structural abnormalities. Unlike control IFM, hinge B-expressing muscle contains asymmetric sarcomeres, with highly variable opposing half-lengths interspersed among homogeneous sarcomeres (Figure 2a–c). Nevertheless, S1 heads of hinge-B myosin are bound to adjacent thin filaments along the length of the “long” half sarcomere (Figure 2d,e). This illustrates that the aberrant sarcomeric dimensions are associated with highly asymmetric thick and thin filaments. *In vitro*, striated muscle myosin self-assembles into inherently asymmetric filaments¹⁶; however, *in vivo* thick filament dimensions in healthy muscle are regulated tightly and are extremely homogeneous^{17–19}. Asymmetric thick filaments (Figure 2a–c) with unperturbed longitudinal S1 spacing along hinge-B myofibrils¹¹ suggest a force imbalance will result *in situ*. Consequently, the unequal distribution of tension apparently damages the neighboring sarcomere (Figure 2a–c). This damage likely influences myofibrillar force production and/or propagation. Additionally, our data suggest that the major filamentous structures and associated proteins of the IFM are highly plastic elements. Since the primary lesion of the mutant sarcomere resides within the myosin rod, it likely affects thick filament polymerization properties and/or length regulation, which correspondingly forces increased thin filament lengths to ensure actomyosin interactions along each half sarcomere. It is interesting to note however that all thick filaments from a single half sarcomere appear to have similar lengths. This suggests certain aspects of normal length regulation are still occurring.

To assess the extent of sarcomeric length inhomogeneity, likely due to thick filament asymmetries, myofibrils were isolated from control hinge-A- or hinge-B-expressing IFM and imaged by confocal microscopy (Figure 3a,b). Individual sarcomere lengths were determined by directly measuring the distance between the centers of adjacent Z-lines directly from fluorescence intensity plots generated from micrographs (Figure 3c). Consistent with previous EM data¹⁰, hinge-B-expressing sarcomeres ($3.48 \pm 0.29 \mu\text{m}$, $n=255$ (mean $\pm$ SD)) containing myosin with aberrantly stiff rods were shown to be ~8% longer than control sarcomeres ($3.23 \pm 0.14 \mu\text{m}$, $n=260$ (mean $\pm$ SD)) (Figure 3d). Moreover, the variances associated with the two groups also differ significantly. It is noteworthy that transgenic hinge-B myosin expression produces sarcomeric asymmetries when myosin throughout the IFM contains identical hinge regions.

Thus, overall sarcomere lengths along hinge-B-containing myofibrils are greater, non-uniform and highly variable relative to those along hinge-A-expressing myofibrils. This suggests that hinge-B is apparently not completely compatible with sarcomere length determining mechanisms when mis-expressed and that myosin rod chemomechanical properties imparted by specific hinge sequences influence thick filament and sarcomeric organization.

We also determined the difference in Z- to M-line distances from opposing halves of individual contractile units to examine the extent of intra-sarcomeric asymmetry (as observed in ultrathin IFM sections; Figure 2a–c). The fractional asymmetry¹⁶ (δ) for hundreds of sarcomeres from myofibrils randomly selected from a population of hundreds of IFM fibers was calculated as the absolute value of one half of the sarcomere length minus the length of the other half, divided by the total length (Equation 1). Hinge-B-containing sarcomeres were shown to have significantly higher δ relative to that from hinge-A-expressing sarcomeres (Figure 3d). Thus, changing the physicochemical properties of a discrete region of the hinge influences the properties of the rod and initiates aberrant thick filament and sarcomere assembly. These inter- and intra-sarcomeric inhomogeneities could disrupt the uniformity of contractile force generation and the efficacy of its transmission along myofibrils, which is consistent with the decreased flight performance of the mutant flies¹⁰.

We extended our analysis to highlight a novel contribution of an S2/LMM hinge mutation to the nanomechanical properties of the human cardiac β -myosin II (*MYH7*) rod and thereby probe its potential role in perturbing sarcomeric organization. Since pure samples of wild-type and mutant human cardiac β -myosin suitable for EM and rod *PL* determination are difficult to obtain, we assessed the mechanical properties of the hinge locally, by performing molecular-dynamics (MD) simulations of a model based on a flexible coiled-coil crystal structure derived from a fragment of human cardiac S2 (PDB code: 2FXM) using CHARMM c33b2 (Figure 4a). We then compared these results to those obtained from an MD simulation of the same model containing the R1193S dilated cardiomyopathy-causing mutation¹². Models examined were centered on residue 1193 (wild-type Arg or mutant Ser) flanked by the neighboring 35 amino acids of the normal rod. The energy minimized structures, differing in only a single residue, had virtually identical shape prior to simulation (Figure 4b, 0 ns). However, during simulation the mutant was considerably stiffer, with its hinge *PL* ~100% greater than the wild-type hinge (65 vs. 33 nm) (Figure 4c). These increases are consistent with markedly increased S2/LMM hinge stiffness and possibly, through propagation along the molecule, with increased overall rod stiffness. As found when a series of amino acids in the *Drosophila* S2/LMM hinge region are replaced *in vivo*, the chemical and physical changes resulting from the *MYH7* hinge mutation could contribute to increased thick filament and sarcomere lengths, length variabilities and asymmetries in diseased human myocardium *in vivo*. Such variations in sarcomeric organization may disrupt efficacy of force generation and transmission to the extracellular matrix. Perturbing these fundamental properties of muscle fibers is likely associated with pathogenic remodeling cascades related to late onset dilated cardiomyopathy in the human patients.

Changing the structural and mechanical properties and altering the physicochemical nanoenvironment of certain regions of the myosin rod (as determined here by measuring *PL* differences) likely influence critical aspects of filament self-assembly and can potentially alter association with ancillary proteins that act as molecular rulers to determine precisely uniform and symmetric filaments²⁰. *In situ*, asymmetric thick filaments and sarcomeres would generate an unequal distribution of tension and yield unidirectional net force^{16,21}. Such an imbalance in force may alter Z-band stress/strain relationships, induce nodal signaling cascades²² and can disrupt neighboring sarcomere structure (Figure 2a–c). Likewise, Z-discs shared by opposing symmetric sarcomeres that differ in overall length may also sense similar stress/strain imbalance and trigger nodal myofiber remodeling cascades²². Our data highlight a new pathogenic pattern for myopathies that result from myosin rod mutations, which like S1 mutations can affect force generation and propagation along myofibrils and therefore possibly share common pathogenic mechanisms. Furthermore, high degrees of sarcomeric asymmetry may be a general hallmark of many skeletal or cardiac myopathies, potentially indicative of disease severity and prognosis and thus a possible index of myofiber dysfunction worthy of future analysis.

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Abbreviation list

S1	subfragment 1
S2	subfragment 2
HMM	heavy meromyosin

LMM	light meromyosin
MD	molecular dynamics
IFM	indirect flight muscle
PL	persistence length
EM	electron microscopy

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Highlights

- Rod disturbances alter myosin's nanomechanical properties
- Altered myosin rod nanomechanics can yield asymmetric myofilaments and sarcomeres
- Asymmetric sarcomeres promote contractile force imbalances
- Disrupted uniformity of force production/propagation could initiate myopathy
- Sarcomeric inhomogeneities might serve as a useful index of myopathic dysfunction

Figure 1.

S2/LMM hinge variants contribute to myosin rod stiffness. *yw* (hinge-A) and *15b-47* (hinge-B) myosin molecules were purified, rotary shadowed and imaged at 23°C with a FEI Tecnai 12 transmission electron microscope operating at 120 kV at a magnification of 21,000X as previously described¹⁰. Digital images were taken with a TVIPS (Tietz) TemCam-F214 high-resolution digital camera. The ~150 nm hinge-A or the ~155 nm hinge-B myosin rods were divided into 3, ~50 nm segments and into 4, ~38 nm segments which were skeletonized after manual selection of points every 4–5 nm along the center of the molecule's longitudinal axis directly from the micrographs. Persistence length (*PL*) was calculated via the tangent correlation method, after θ (the deviation angles along the myosin rod from an idealized straight rod) was determined for each tail segment. Previously developed algorithms to determine *PL* and θ were used^{13–15}. Plots relating the inverse slope of $\ln \langle \cos(\theta(s)) \rangle$ to the segment length yielded the *PL* values, where the factor of 2 in $\langle \cos(\theta(s)) \rangle = e^{-s/2(PL)}$ accounts for the two-dimensionality of electron micrographs. (a) Transgenic *Drosophila* myosins that differ only in 19 amino acids within hinge-A or hinge-B (A/B) of the S2/LMM hinge were expressed in the IFM. The limited myosin proteolysis products known as sub-fragment 1 (S1), sub-fragment 2 (S2), heavy meromyosin (HMM) and light meromyosin (LMM) as well as the approximate location of “skip bends” are indicated²³. 1st, 2nd, 3rd or 4th refer to the various rod sections that were independently evaluated in each segmental-based analysis. *PL* values (in nm) were calculated for each segment for hinge-A and hinge-B expressing molecules. (b) Myosin rod segment persistence lengths (*PL*) were determined from rotary shadowed images of hinge-A-expressing (top row) and of hinge-B-expressing (bottom row) molecules. (c) The tangent correlation method used to calculate *PL* plots $\ln \langle \cos(\theta(s)) \rangle$ as a function of arc length along the samples (where θ is the deviation angle from a straight rod). Measurements from rotary shadowed molecules suggest hinge-B rods are less flexible than hinge-A rods. *PL* measurements reveal minor rigidity increases of the initial 50 nm (first one-third) and of the 2nd quarter of hinge-B rods consistent with a local stiffening contribution from the transgenic hinge. The remaining downstream segments of hinge-B rods are substantially more rigid relative to the same fragments of hinge-A tails. This suggests propagation of the mechanical effects of amino acid substitutions in the hinge region, which lead to overall stiffness increases along hinge-B expressing myosin rods.

Figure 2.

Increased myosin rod stiffness is associated with sarcomeric disorganization. Indirect flight muscle (IFM) myofibrils from ~2 week old flies were prepared for transmission electron microscopy according to. IFM membranes were glycerinated to allow visualization of myosin heads in rigor²⁶. Following fixation, block staining, dehydration, infiltration and embedding of IFM, ultra-thin sections (25–40 nm) were cut with a Diatome diamond knife, picked up on carbon coated grids, stained and rinsed. Sections were photographed on S0163 film using a FEI 420 electron microscope operating at 100 kV. Images were acquired at 3,300X (Figure 2a,b), 10,500X (Figure 2c) and at 18,500X (Figure 2d,e). Negatives were digitized at a resolution of 800 dpi using a flatbed scanner. (a,b) Electron micrographs of hinge-B-expressing myofibrils reveal highly irregular sarcomeres. Asymmetric force production apparently damages neighboring sarcomeres (*). This damage likely influences myofibrillar tension generation and transmission. (c) Some half sarcomeres (enlarged from (b)) were nearly twice as long as others. (d,e) Enlarged regions from the long (left) half of the asymmetric sarcomere in (c) that are immediately adjacent to the Z-disc (d) or to the M-line (e) reveal myosin heads bound in rigor to neighboring actin filaments. This illustrates myosin polymerized into asymmetric thick filaments that run nearly the entire length of each half sarcomere.

Figure 3.

Determination of sarcomeric dimensions along myofibrils expressing different S2/LMM hinges. Fluorescent labeling of IFM myofibrils was carried out according to standard procedures^{27–29}. Briefly, hemi-thoraces from ~20 (2 day old) female *pwmhc2* (transgenic hinge-A) or *15b-47* (transgenic hinge-B) flies were fixed, gently homogenized, blocked, stained with primary (anti-skeletal muscle myosin antibody (D. Kiehart)) and secondary antibody plus fluorescently labeled phalloidin (goat anti-rabbit Cy5 antibody (Sigma-Aldrich) and Phalloidin-TRITC (Sigma-Aldrich)) and rinsed. ~5 hemi-thoraces were carefully transferred to a drop of Vectashield (Vector Laboratories, Inc.) flanked by 2 coverslips on a microscope slide. Individual IFM fibers were cut from the hemi-thoraces using microsurgical scissors and lightly minced to help spread myofibrils. A third coverslip was placed on top of the other 2 to protect the underlying myofibrils from stretching. The samples were imaged at 100X magnification with a Leica IRBE confocal microscope. Fluorescence intensity plots of 3–4 consecutive sarcomeres were generated along the center of the long axis of randomly selected images of equally stretched hinge-A or hinge-B myofibrils using Leica Confocal Software. For total sarcomere length the distance from the center of each minimum peak for actin staining (Z-line) to the center of the next peak minimum was measured directly from plots. A Student's t-test was used to determine if significant differences in the mean $\pm$ SD values were present between the two groups. F-tests were performed to determine if the variances among the two populations differed significantly. To assess the extent of intra-sarcomeric inhomogeneity, fractional asymmetry was calculated as in Brown *et al*¹⁶, with minor modifications. Z- to M-line distances from opposing halves of individual contractile units were determined directly from fluorescence intensity plots of myofibrils by measuring the distance from the center of the actin peak minimum signal (Z-line) to the center of myosin's peak maximum staining (M-line). Values for each sarcomere were calculated as (Equation 1):

$$|DL_{(Z-M)} - DR_{(Z-M)}|/SL_{tot}$$

where $DL_{(Z-M)}$ = Z- to M-line distance on the left half of a sarcomere; $DR_{(Z-M)}$ = Z- to M-line distance on the right half of a sarcomere; SL_{tot} = total sarcomere length. A Student's t-test was used to determine if significant differences in the mean $\pm$ SD values were present between the two groups. (a,b) Confocal micrographs of hinge-A (a) and hinge-B (b) myofibrils were used for quantifying sarcomere length and inter- and intra-sarcomeric length variability. Actin is displayed in red and myosin (M-line) in cyan. (b, bottom) An enlarged image of a single hinge-B sarcomere displaying typical asymmetry. The arrow delineating actin staining on the left half of the sarcomere is 1.72 μ m while that on the right is 1.48 μ m. (c) Fluorescence intensity plots were generated from confocal micrographs of IFM myofibrils. Fluorescence intensity, from channels displaying actin-associated (red) and myosin- (M-line) associated (cyan) fluorescence was plotted relative to horizontal distance along myofibrils isolated from hinge-A- or hinge-B-expressing flies. Sarcomere lengths were determined by measuring Z-line to Z-line distances while fractional asymmetry was determined according to Equation 1. Effects of transgene expression or artifacts that may otherwise skew our measurements are controlled by complementary analysis of transgenic

controls expressing hinge-A myosin. (d) Hinge-B sarcomeres ($3.48 \pm 0.29 \mu\text{m}$, $n=255$) were significantly longer ($***p < 0.0001$) than hinge-A sarcomeres ($3.23 \pm 0.14 \mu\text{m}$, $n=260$) and the variances associated with the two groups also differ significantly ($p < 0.0001$). Hinge-B sarcomeres also exhibited significantly greater fractional asymmetry relative to hinge-A sarcomeres (0.014 ± 0.011 , $n=255$ vs. 0.010 ± 0.008 , $n=260$, $***p < 0.0001$).

Figure 4.

Molecular-dynamics (MD) simulations of an S2/LMM hinge with a human cardiomyopathy mutation. MD simulations were carried out starting with initial models based on a flexible coiled-coil crystal structure derived from a fragment of human cardiac β -myosin II S2 (PDB code: 2FXM). The models are essentially straight. They were comprised of the wild type (R) or the mutant (S) residue at position 1193 flanked by the same ~35 amino acids of the MYH7 myosin rod. The initial structures were energy-optimized and the MD simulations were run for 20 ns at 300°K with Langevin dynamics and an implicit solvent model using the program CHARMM c33b2 as described^{13–15}. The persistence length (PL) for each model was determined via tangent correlation analysis from θ (deviation angles of individual MD conformers from an idealized straight rod). PL from MD simulations is calculated without the factor of 2 (see above) since the simulations contain 3-D data. (a) Models used in MD simulations were based on a coiled-coil crystal structure derived from a fragment of human cardiac β -myosin II S2 (PDB code: 2FXM) and were comprised of the wild-type (R) or the mutant (S) residue at position 1193 (red) flanked by identical amino acids of the rod. (b) Center line plots of 2D projections of structures averaged over 1 ns intervals during the 20 ns simulation (top wild-type, bottom mutant) generated. The structures are displayed at the indicated times sampled. (c) Plots used to determine the persistence length (PL) from the

MD data, using the tangent correlation method. Measurements of the MD simulations suggest the mutation induces a ~100% increase in *PL* (33 to 65 nm) and thus substantially stiffens the myosin rod's S2/LMM hinge.