Molecular consequences of the R453C hypertrophic cardiomyopathy mutation on human β-cardiac myosin motor function

Ruth F. Sommese, Jongmin Sung, Suman Nag, Shirley Sutton, John C. Deacon, Elizabeth Choe, Leslie A. Leinwand, Kathleen Ruppel, and James A. Spudich

Dep. of Biochemistry, Dept. of Pediatrics (Cardiology), and Cancer Biology Program, Stanford University School of Medicine, Stanford, CA 94305; Dep. of Applied Physics, Stanford University, Stanford, CA 94305; and BioFrontiers Institute, Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO 80309

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Cardiovascular disorders are the leading cause of morbidity and mortality in the developed world, and hypertrophic cardiomyopathy (HCM) is among the most frequently occurring inherited cardiac disorders. HCM is caused by mutations in the genes encoding the fundamental force-generating machinery of the cardiac muscle, including β-cardiac myosin. Here, we present a biomechanical analysis of the HCM-causing mutation, R453C, in the context of human β-cardiac myosin. We found that this mutation causes a ∼30% decrease in the maximum ATPase of the human β-cardiac subfragment 1, the motor domain of myosin, and a similar percent decrease in the in vitro velocity. The major change in the R453C human β-cardiac subfragment 1 is a 50% increase in the intrinsic force of the motor compared with wild type, with no appreciable change in the stroke size, as observed with a dual-beam optical trap. These results predict that the overall force of the ensemble of myosin molecules in the muscle should be higher in the R453C mutant compared with wild type. Loaded in vitro motility assay confirms that the net force in the ensemble is indeed increased. Overall, this study suggests that the R453C mutation should result in a hypercontractile state in the heart muscle.


The authors declare no conflict of interest.

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1To whom correspondence may be addressed. E-mail: js499@stanford.edu or kruppel@stanford.edu.

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moiety (SI Materials and Methods). We refer to this construct as simply human β-cardiac S1 throughout. Here we present a description of the effects of this mutation on human β-cardiac S1 function at both the single-molecule and ensemble levels. Single-molecule analysis revealed a significant increase in intrinsic force of the mutant motor by 50%, such that the net ensemble force is higher than the wild-type (WT) motor, as confirmed by a loaded in vitro motility assay. Together, the data support a model whereby heart muscle containing this mutation would be hypercontractile.

Results

R453C Human β-Cardiac S1 Has a Lower Maximum Actin-Activated ATPase than WT. To measure the maximal rate of ATP turnover ($k_{cat}$) by the acto–myosin complex, we used an actin-activated myosin ATPase assay at 23 °C. The $k_{cat}$ of R453C human β-cardiac S1 (5.0 ± 0.2 s$^{-1}$) was significantly lower (30%; $P < 0.0002$) than that of the WT motor (7.4 ± 0.4 s$^{-1}$) (Fig. 1, Table S1). This difference was also seen with human β-cardiac S1 that was freshly recycled by binding to actin in the absence of ATP and then releasing active heads from the actin in 2 mM ATP (this process eliminates “dead heads” from the population). The $K_m$ for WT was 38 ± 4 μM and for R453C was 28 ± 4 μM.

The inverse of the $k_{cat}$ is the amount of time it takes for the motor to complete one ATPase cycle, $t_c$ (Fig. 1B). Thus, the $t_c$ for WT human β-cardiac S1 was 140 ± 10 ms and for R453C was 200 ± 10 ms. These measures of $t_c$ point to a fundamental difference in the crossbridge kinetics between R453C and WT human β-cardiac myosin.

R453C and WT Human β-Cardiac S1 Have a Similar Stroke Size and Strongly Bound State Time in the Optical Trap. We measured the stroke size ($d$) of WT and R453C human β-cardiac S1 at the single-molecule level using a dual-beam laser trap assay at 23 °C. The magnitude of the displacement of the optically trapped bead at low trap forces is a measure of the stroke size ($d$) of the myosin produced during the power stroke (Fig. S2). There was no statistical difference between the stroke sizes of R453C (6 ± 1 nm) and WT (6 ± 1 nm) human β-cardiac S1. These values are consistent with a rotation of ~70° of a truncated lever arm (consisting of only one light chain).

It was also possible to get an estimate of the length of time that the myosin molecule is strongly bound to the actin filament in saturating ATP concentration, or the strongly bound state time ($t_s$) (Fig. S2). For the R453C mutant and the WT, $t_s$ was ~16 ms. These values are, however, only estimates given the difficulty of accurately detecting and selecting short events in the trap, which are challenging to distinguish from Brownian noise of the trapped beads and instrument noise. Hence, a small change in the $t_s$ is currently difficult to measure with our instrument.

R453C Human β-Cardiac S1 Has a Lower Velocity than WT. We measured the velocity of motor-driven actin filaments in methylcellulose as a function of actin filament length for both WT and R453C human β-cardiac S1. The average maximum velocity ($v_o$) for R453C at 23 °C for human β-cardiac S1 (610 ± 30 nm·s$^{-1}$) was significantly slower than that of WT (800 ± 40 nm·s$^{-1}$) ($P < 0.0001$). The decrease in velocity for R453C compared with WT was also observed at 30 °C (Fig. 2, Table S1).

The maximum velocity $v_{fi}$ in the in vitro motility assay is related to the displacement generated by the myosin power stroke (i.e., the stroke size $d$) and the duration that myosin remains strongly attached to actin ($t_s$), such that $v_{fi} = d/t_s$ (15). Besides the estimates from the trap measurements, the $t_s$ of S1 in saturating ATP concentration can be estimated by fitting the velocity as a function of actin filament length as shown in Fig. 2 (15). Assuming $d$ is ~6 nm from the trap data, $t_s$ should be ~10 ms for R453C and ~7 ms for WT human β-cardiac S1 from the $v_o$ measurements at 23 °C. With this change in $t_s$ and the similar increase in $t_c$, the fraction of heads bound to actin at steady-state (i.e., the duty ratio $= t_c/t_s$) is similar between R453C and WT. We note that this is the observed duty ratio at saturating ATP and saturating actin concentrations. The duty ratio is ~5–10%, which is expected for β-cardiac myosin, as it is a low duty ratio motor (16, 17).

R453C Human β-Cardiac S1 Has a Significantly Increased Intrinsic Force Compared with WT. In addition to the mutant’s effect on motility, we investigated whether the force-generating capability of the myosin molecule was affected, using a dual-beam optical trap. Here we determined the motion of the trapped beads with high spatial and temporal resolution under high load, achieved by a position feedback system. This feedback signal moves the trap position in response to the myosin stroke and can be used to quantify the intrinsic force ($f$) generated by a single myosin molecule. For the R453C mutant, the average intrinsic force produced by a single myosin molecule was 2.1 ± 0.1 pN, which was significantly increased compared with WT (0.4 ± 0.1 pN). Together, the data support the hypothesis that the R453C mutation causes a hypercontractile phenotype in human heart muscle.
In the context of the heart, individual cardiac myosin motors work as an ensemble to generate a contractile force. The maximum unloaded in vitro velocity ($v_{\text{exp}}$) and the actin length ($l$) to the following equation: $v_{\text{exp}} = \frac{d}{t_{\text{exp}}} \left[1 - \left(1 - \frac{d}{l}ight)^{\frac{1}{2}}\right]$. The $d$ values are from our optical trap measurements (6 nm), and the $t_{\text{exp}}$ values are from our actin-activated ATPase measurements. The fitting parameters are $k$, which represents the number of motor heads per unit length of actin filament, and $t_{\text{exp}}$. The maximum unloaded in vitro velocity ($v_0$) is equal to $d/t_{\text{exp}}$. 50% higher ($P < 0.001$) than the force generated by the WT human β-cardiac S1 under the same conditions (1.4 ± 0.1 pN) (Fig. S3).

**The Ensemble Force of R453C Human β-Cardiac S1 Is Significantly Higher than WT.** In the context of the heart, individual cardiac myosin motors work as an ensemble to generate a contractile force that propels blood throughout the circulatory system. The ensemble force should be the intrinsic force ($f$) multiplied by the number of myosin heads interacting with the actin in a force-producing state at any moment, which is determined by the duty ratio ($t_{\text{on}}/t_{\text{cycle}}$). That is, $F_{\text{ensemble}} = f \cdot t_{\text{on}}/t_{\text{cycle}} \cdot N_T$, where $N_T$ is the total number of heads in the overlap zone of the sarcomere. We therefore tested the effect of the R453C mutation on myosin motor function under load using an ensemble of motors. Specifically, we used the in vitro motility assay in the presence of an external load to examine whether the R453C mutant’s ensemble force-generating ability was altered. We introduced a frictional load in the in vitro motility assay using α-actinin, an actin-binding protein that leads to a molecular tug of war with myosin (18, 19). Typically, the amount of α-actinin needed to stop filament movement is reported as an indication of the myosin isometric force. As shown in Fig. 3B, the R453C mutant needed more α-actinin than WT to stop the same fraction of filaments from moving. Recognizing that the best model to describe such data is still under debate (18), we restrict ourselves to a qualitative description of the difference between R453C and WT. Nevertheless, these ensemble force measurements show that R453C has a higher force-generating ability compared to WT and corroborates our single-molecule intrinsic force measurements.

**Discussion**

Although the clinical aspects of HCM have been studied for many decades, there is no clear understanding of the underlying molecular mechanisms leading to the disease. In the case of R453C, the molecular data have not been consistent across studies. Palmer et al. (13) showed a decreased $k_{\text{cat}}$ when the mutation was in mouse α-cardiac myosin. Debold et al. (14) later found with mouse α-cardiac myosin no change in $k_{\text{cat}}$ or $v_{\text{cat}}$, but an increase in the average ensemble force ($F_{\text{ensemble}}$). These inconsistencies are likely due to both the isoform backbone and source of the purified cardiac myosin. Mouse α-cardiac myosin differs by 83 residues in the head domain (human residues 1–808) from human β-cardiac myosin. When one is considering the effects of a single residue change on the function of human β-cardiac myosin, working with myosins that contain >80 residue differences is far from ideal.

By using a recently developed myoblast-based cardiac myosin expression system (11) and focusing on purified human β-cardiac myosin, we are now examining the effects of HCM and dilated cardiomyopathy (DCM) mutations in the very protein in which the diseased state is produced. Sata et al. (12) achieved some expression of human β-cardiac myosin in SF9 cells, but with considerably lower ATPase activities than we report here for both WT and R453C β-cardiac myosin. Their large decreases in both ATPase and in vitro motility velocities for the R453C mutant compared with WT, as well as for other mutations they studied, likely reflect the difficulty of obtaining active recombinant human...
β-cardiac myosin in heterologous expression systems, possibly due to the lack of muscle-cell–specific chaperones (11).

The ~30% decreases that we observed in both the \( k_\text{cat} \) of the ATPase and the unloaded maximum velocity of movement of the R453C mutant S1 compared with the WT S1 result in corresponding increases in both \( t_\text{s} \) and \( t_\text{t} \) (Fig. 1B). The duty ratio, \( t_\text{t}/t_\text{s} \), which is one determinant of the total ensemble force production by the muscle, is therefore not changed much in the R453C β-cardiac myosin (~5% lower than WT). The major change in the human β-cardiac S1 as a result of the R453C mutation is a ~50% increase in intrinsic force, which should lead to hypercontractile cardiac function.

It is important to point out that the duty ratio estimated in this study is the unloaded duty ratio. During contraction, though, muscle is under load and load affects the kinetics of muscle mechanics (Fenn Effect) (20). One recent study with smooth muscle myosin was able to examine the effect of load on the \( t_\text{s} \) using an optical trap (21). One difficulty with measuring \( t_\text{s} \) for cardiac myosin, though, is that the \( t_\text{t} \) under saturating ATP concentrations is extremely short and difficult to detect within the noise of the trap. An accurate measurement of the \( t_\text{t} \) under varying load will be key to understanding the effects of different β-cardiac human myosin mutations on muscle function and power.

It is possible that the R453C mutation alters the “spring constant” \( k \) of the motor, simplifying the intrinsic force as \( f = k \cdot x \), where \( x \) is the displacement due to the stroke. It is interesting that residue 453 lies very near strand five of the β-pleated sheet in the core of the myosin head domain (Fig. 4). It is interesting to note that there are no hypertrophic, dilated, or left ventricular non-compaction cardiomyopathy-causing mutations that have been identified in this strand of the β-pleated sheet (there have been numerous mutations found in the other six strands). It is possible that any alterations to this strand may have significant enough consequences on motor function to be nonviable. This β-pleated sheet, also referred to as the transducer, undergoes a distortion or twist during the transition from the prestroke to poststroke states (22). The strain in this sheet may well be part of the spring that establishes the intrinsic force-producing capability of the myosin head. R453C lies at the bend between an α-helix and the 7-stranded sheet, and an increase in stiffness at the bend would contribute to a larger spring constant. The overall spring constant must of course be related to the overall changes in the motor structure when transitioning between the prestroke and poststroke states.

An early hypothesis regarding HCM pathophysiology was that HCM-causing mutations such as R453C resulted in decreased contractile activity, and hence the hypertrophy of the cardiac muscle was a compensatory response. This hypothesis appeared to be corroborated by initial biochemical studies of HCM-causing β-cardiac myosin mutant proteins (7, 12, 23). As mentioned above, however, these results likely reflected the difficulty of obtaining active motor from small amounts of biopsy material or from heterologous expression systems. More recent studies of muscle fibers, cardiac myofilibrils, or cardiac myosomes containing various HCM mutations reveal a picture more consistent with these mutations resulting in enhanced contractility (reviewed in ref. 6), in agreement with the results that we report here for R453C β-cardiac myosin. It has been noted that patients with HCM often have enhanced contractile function, measured echocardiographically as an increased left ventricular ejection fraction (LVEF) (24, 25). Recent echocardiographic studies of patients harboring HCM-causing mutations in β-cardiac myosin who have no evidence of hypertrophy (so-called genotype positive/phenotype negative or preclinical HCM patients) demonstrate that these individuals also have increased LVEFs. This suggests that the enhanced contractility is due to the mutations themselves and not a result of the muscle hypertrophy (26). The mechanism by which this hypercontractility could serve as a stimulus for hypertrophy is not known.

This study demonstrates enhanced function of highly purified, homogeneous human β-cardiac myosin containing an HCM-causing mutation. More studies on the biochemical and biophysical properties of a variety of HCM mutations studied in the human myosin context are needed to see whether this will prove to be more generally true. A future challenge will be to connect the underlying biochemical and biophysical mechanisms to the subsequent pathophysiological changes observed in patients carrying HCM mutations.

Materials and Methods

Myosin Constructs and Protein Expression. The myosin proteins used in this study were constructed and produced as previously described (11, 27), using the AdEasy Vector System (Qbiogene, Inc) with minor modifications. MYH7 cDNA and myosin light chain 3 (MYL3) (ventricular ELC) were purchased from Open Biosystems (Thermo). A truncated version of MYH7 (residues 1–808) was made, corresponding to a short S1. For construct details, see SI Materials and Methods: Replication-deficient recombinant adenoviruses were produced and amplified in HEK29293 cells. Viral particles were purified from clarified cell lysates by sequential step and linear cesium chloride gradients, and concentrated virus was stored in a glycerol buffer at ~20 °C.

Murine C57BL/6 myoblasts (ATCC) were cultured in GlutaMAX DMEM (Gibco) supplemented with 10% (vol/vol) FBS (Sigma) and 1% penicillin/streptomycin (Gibco). Forty-eight hours postdifferentiation in DMEM supplemented with 2% (vol/vol) horse serum (Sigma) and 1% penicillin/streptomycin, cells were infected with 1 × 10^5–1 × 10^6 plaque-forming units of both MHC and ELC viruses. Three to four days postinfection, myotubes expressing recombinant MHC were collected by cell scraping and lysed. FLAG-tagged ELC and associated MHC was purified from clarified lysate with anti-FLAG resin. The human β-cardiac S1 was separated from endogenous skeletal myosin and further purified by ion exchange chromatography on a 1 mL HiTrap Q HP column (GE Healthcare) (Fig. 51).
Additional Protein Purification. Actin was prepared from fresh chicken breast skeletal muscle as previously described with slight modification (28). In the polyclonal to antibodies methods as described in SI Materials and Methods (30). For the loaded in vitro motility assays, actin was purified from chicken gizzards as previously described with a few minor changes (31). After the diethylaminoethanol (DEAE)-Sepharose and S-Sepharose steps described, the protein was bound to a hydroxyapatite column and eluted with a 0–250 mM potassium phosphate gradient. Binding to actin filaments was confirmed by sedimentation analysis.

Actin-Activated ATPase Assay. For the ATPase, gelsolin was added to actin at a ratio of 1:1,000. Gelsolin at this concentration acted to decrease the viscosity of the actin, and thereby decrease pipetting error, without interfering with the ATPase activity (Fig. 54). Actin-activated ATPase assays were then performed as previously described using a colorimetric readout (32). Briefly, motor and beads were diluted to 510. The concentration of 0.03–0.1 μM (with 3–5 times as much for the basal myosin ATPase control to amplify the signal) with 2 mM ATP (CalBiochem) and actin at concentrations ranging from 0 to 100 μM. The final buffer conditions were 10 mM imidazole, pH 7.5, 5 mM KCl, 3 mM MgCl2, and 1 mM DTT. The reaction was performed at either 23 °C or 30 °C with shaking in a Thermo Scientiﬁc Multiskan GO, and four to five time points were taken for each concentration. As the cardiac S1 activity was linear over the time period of the assay, an ATP-regenerating system was not necessary. Basal activity (<0.2 s−1) was subtracted to get actin-activated ATPase activity. Data were fit to a Michaelis–Menten equation to determine the maximal activity (kcat) and the associated actin constant for myosin (Km) using OriginLab. Statistical analysis to determine significance was performed using a standard Welch’s t test. All of our P values were < 0.01.

Unloaded in Vitro Motility. The basic method followed our previously described motility assay (33) with the following modifications. Coverslips were pretreated with nitrocellulose (1% in amyl acetate, Ernst Fullam) diluted to 0.2% in amyl acetate (Sigma). Before blocking of the surface with 1 mg/mL BSA, anti-GFP antibody (Millipore) was flowed in to coat the coverslip such that β-cardiac myosin constructs could be attached to the coverslip by way of their C-terminal EGFP tag. The assay buffer (AB) used was 25 mM imidazole (pH 7.5), 25 mM KCl, 4 mM MgCl2, 1 mM EGTA, 1 mM DTT. Both anti-GFP antibody and myosin concentrations were varied to determine the surface density that supported maximal velocity. The final motility solution of AB with 1 mg/mL BSA (ABBSA) contained methocellulose at a concentration of 0.5% (33), tetramethylrhodamine (TMR)-phalloidin labeled actin, 2 mM ATP, an oxygen-scavenging system (0.3–0.4% glucose, 0.25 mM glucose oxidase, 0.45 mM catalase), and an ATP regeneration system [1 mM phosphocreatine (PCR), 0.1 mM creatine phosphokinase (CPK)]. Movies were obtained at 23 °C and 30 °C at a frame rate of 1 Hz using a Nikon Ti inverted microscope with Andor iXon+EMCCD camera model DU885. For discussion regarding analysis of velocities and motor cleanup, see SI Materials and Methods.

Loaded in Vitro Motility. Loaded in vitro motility assays were performed using α-actinin as a load (34). After anti-GFP antibody was incubated with the surface, excess antibody was washed out with AB, followed by the desired α-actinin concentration. The surface was then blocked with BSA and motor flowed in as described above. For each condition, multiple 30-s movies were taken in each flow cell and movies were then analyzed to determine the fraction of filaments moving during those 30 s. The concentration of motor and anti-GFP antibody was held constant for each α-actinin concentration. Measuring the fraction of filaments stuck proved to be the most consistent way of measuring the effect of α-actinin on filament movement, as has been previously observed (34).

Optical Trap. Experiments were performed in a manner similar to those described for the unloaded in vitro motility assay. Typical myosin concentrations used were ~200 pM to ensure binding events from a single myosin molecule. ABBSA containing ATP, TMR-phalloidin labeled bimot–actin filaments, streptavidin-coated polystyrene beads, 1 mM phalloidin, and the oxygen-scavenging and ATP regeneration systems described above was flowed through the chamber. The chamber was sealed with vacuum grease, and each such slide was used up to 1 h.

Detailed Experimental Setup, experimental procedure, and the data analysis are described in SI Materials and Methods. Briefly, each beam trapped a streptavidin-coated polystyrene bead (diameter of 1 μm) that was attached to the ends of a fluorescently labeled bimot–actin filament. The bead–actin–bead system, called actin dumbbell, was tightly stretched and thereby decreased pipetting error, without interfering with the ATPase activity. The basic method followed our previously described motility assay (33) with the following modifications. Coverslips were pretreated with nitrocellulose (1% in amyl acetate, Ernst Fullam) diluted to 0.2% in amyl acetate (Sigma). Before blocking of the surface with 1 mg/mL BSA, anti-GFP antibody (Millipore) was flowed in to coat the coverslip such that β-cardiac myosin constructs could be attached to the coverslip by way of their C-terminal EGFP tag. The assay buffer (AB) used was 25 mM imidazole (pH 7.5), 25 mM KCl, 4 mM MgCl2, 1 mM EGTA, 1 mM DTT. Both anti-GFP antibody and myosin concentrations were varied to determine the surface density that supported maximal velocity. The final motility solution of AB with 1 mg/mL BSA (ABBSA) contained methocellulose at a concentration of 0.5% (33), tetramethylrhodamine (TMR)-phalloidin labeled actin, 2 mM ATP, an oxygen-scavenging system (0.3–0.4% glucose, 0.25 mM glucose oxidase, 0.45 mM catalase), and an ATP regeneration system [1 mM phosphocreatine (PCR), 0.1 mM creatine phosphokinase (CPK)]. Movies were obtained at 23 °C and 30 °C at a frame rate of 1 Hz using a Nikon Ti inverted microscope with Andor iXon+EMCCD camera model DU885. For discussion regarding analysis of velocities and motor cleanup, see SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

Myosin Constructs. For expression of human β-cardiac subfragment 1 (S1)–eGFP fusion proteins, AscI and HindIII restriction sites and an N-terminal GSG linker were added to the gene encoding eGFP (1) using PCR amplification. The product was ligated into pShuttle–CMV that had been modified by PCR to contain AgeI and AscI restriction sites between the existing KpnI and HindIII sites in the multiple cloning site. Myosin heavy chain 7 or MHY7 cDNA encoding residues 1–808 of the β-cardiac myosin heavy chain with or without the R453C mutation had flanking AgeI and AscI restriction sites added by PCR amplification and were inserted into pShuttle–CMV downstream of the CMV promoter and upstream and in-frame with a GAPGSG linker–eGFP fusion construct. For expression of the essential light chain (myosin light chain 3, or ELC), full-length human gelsolin (2) was expressed and purified as described by others (5). Track velocities in each surface (50–150 filaments) were determined using Matlab FIESTA software (4), followed by threshold scoring based on maximum change in filament length (1 μm), maximum change in angle (90°), minimum and maximum velocity, and number of consecutive frames of movement (5 frames at 1 Hz). Manual analysis was originally performed on >70% of surfaces, before reanalysis of all surfaces by threshold analysis. Threshold and manual analysis agreed in the overall results, but threshold analysis was preferred as it took out manual bias, as has been discussed by others (5). Track velocities in each surface (50–150 filaments) were fit to an equation for the length dependence of filament velocity (6). For both motors, multiple surfaces and conditions for five or more preps were examined and two to four preps with the fastest and smoothest velocities were used to determine the average velocity.

For myosin II motility, it is common practice to treat the surface with unlabeled actin to block inactive motors (7). We found that this was not sufficient to block dead motors, and at high enough concentrations, this “dark” actin will in fact slow surface velocities. Instead, we had stringent quality standards for preparation of the motor. Two methods were used to eliminate inactive or damaged S1 heads that otherwise slowed down motility. One method involved cycling the motor by binding to actin in the absence of ATP, sedimenting the bound motor with actin, releasing active motor with ATP, and then removing the actin by sedimentation. A second well-known method is termed “dead-heading,” in which we precipitated irreversible rigor heads by adding actin in the presence of ATP and then sedimenting the actin. This was performed multiple times until all filaments are moving smoothly on a surface and no stuck or slowed filaments were observed. Stringent requirements for motility were critical to obtain the fastest speeds as a small proportion of rigor motor easily slowed speeds >10%.

Gelsolin Protein Expression. Full-length human gelsolin (2) was expressed and purified based on previous methods (3). Briefly, BL21 DE3 cells were transformed, grown, induced, pelleted, resuspended in PBS, and frozen in liquid nitrogen for later use. Cells were resuspended in gelsolin buffer A (3) for 30 min at 4 °C, and then lysed by sonication. The lysate was centrifuged for 45 min at 32,000 × g in a JA-20. Ammonium sulfate was slowly added with stirring to lyse at 4 °C to 75% (47.5 g per 100 mL). The lysate was stirred an additional 15 min before centrifugation for 20 min in a JA-20 at 8,000 × g rpm. The pellet was resuspended in gelsolin buffer B (3) and dialyzed against the same buffer overnight. Gelsolin was then purified over a 5 mL HiTrap Q FF column (GE Healthcare) equilibrated in buffer B with a 0–500 mM NaCl gradient. Gelsolin fractions were dialyzed into buffer C (3), clarified to remove precipitation, and then run over a 5 mL HiTrap S FF column (GE Healthcare) in a similar manner as above. Final fractions of gelsolin were dialyzed into buffer D (3), flash frozen in liquid nitrogen, and stored at −80 °C.

Motility Analysis and Motor Cleanup. Tracks were identified using Matlab FIESTA software (4), followed by threshold scoring based on maximum change in filament length (1 μm), maximum change in angle (90°), minimum and maximum velocity, and number of consecutive frames of movement (5 frames at 1 Hz). Manual analysis was originally performed on >70% of surfaces, before reanalysis of all surfaces by threshold analysis. Threshold and manual analysis agreed in the overall results, but threshold analysis was preferred as it took out manual bias, as has been discussed by others (5). Track velocities in each surface (50–150 filaments) were fit to an equation for the length dependence of filament velocity (6). For both motors, multiple surfaces and conditions for five or more preps were examined and two to four preps with the fastest and smoothest velocities were used to determine the average velocity.

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Optical Trap Setup. The dual-beam laser trap techniques used in our experiments are described in detail elsewhere (8). Briefly, a 1,064 nm laser light from a diode pumped Nd:YAG laser (YLR-10–1064-LP, IPG Photonics) is split into two beams using a half-wave plate followed by a polarizing beam splitter. A high numerical aperture (NA = 1.45) oil immersion objective lens (TIRF, CFI Plan Apo, 60x, Nikon) focuses the two laser beams to produce two optical traps. Bead displacement was detected with a back-focal plane interferometry method (9) by using a separate 845 nm detection laser beam (LU0845, Lumics) and an IR-enhanced quadrant photodiode detector (QPD, QP45-QHVSD, Pacific Silicon Sensor). Signal from the QPD was sampled at 40 kHz through a data acquisition card (PCIe-6363, National Instrument).

ADP Release Rate Measurement with the Optical Trap. At saturating ATP concentration, ADP release rate determines the strongly bound state time (t). We used the optical trap assay to measure the t from the single myosin molecules. A detailed description of the analysis method used in our experiments is described elsewhere (8). Briefly, we selected the binding events from the mean displacement of the bead position, the covariance of each bead position, and the cross-variance between the two bead positions. Such selection of independent binding events resulted in a dwell time histogram as shown in Fig. S2B. Because the ADP release rate is a first-order kinetic process, the dwell time distribution is an exponential. The noise in our experimental system, however, limits the selection of very short binding events, typically less than ~10–20 ms. Therefore, instead of using a simple exponential, we used an exponential function with a cutoff followed by a convolution with a Gaussian function to fit the data. We used a maximum likelihood estimation to extract the strongly bound state time. An example of the fitting result is shown as a solid line in the Fig. S2B.

Intrinsic Force Measurement with the Optical Trap. To measure the intrinsic force produced by a single myosin molecule, we used a position feedback control in which the quadrant photodiode output was fed into the driver of the acousto-optic deflector (AOD, IntraAction) to maintain the bead’s position in place. A detailed isotropic force measurement procedure is described elsewhere (10–12). The position of one of the traps was controlled with the AOD. The trap to control was chosen to be applying the load against the direction of the motor stroke to maintain the tension in the actin dumbbell. Using a feedback control program (written in LabView), one microsphere was held stationary by rapidly deflecting the trap with the AOD.
Isometric force is measured as a product of trap stiffness and the displacement of the deflected trap beam. The response time of our feedback system is $\sim 10$ ms, which is not fast enough to immediately respond during the power stroke. Therefore, all force measurements were carried out at low ATP concentration (typically $\sim 500$ nM) to ensure long binding time ($>1$ s) (Fig. S3A). Clear force events from individual molecules were selected and binned into a force histogram. Due to the noise in the data, small forces below $0.5$ pN were not collected efficiently. The number of total force events from individual molecules on average was more than 50. Because the small binding events were not sufficient to obtain good statistics, we combined the force events in each preparation, typically two or three molecules, to have enough data for the analysis. We used a Gaussian force distribution with a cutoff to take into account the missing small forces. A least square fitting with the histogram resulted in a mean force around the peak value. We have noticed that the force distributions show long tail or a second peak in a higher force regime. These high-force events are a small fraction, but they can easily alter the fitting results and the interpretation of the data. To extract the mean force from the main population around the peak value, we instead used a double Gaussian distribution (Fig. S3 B and C). Identification of the second population is interesting and can be a future study. One possibility is that they might be related to a different molecular state (e.g., closed nucleotide pocket) (13).


Fig. S1. Expression of recombinant human β-cardiac S1. SDS/PAGE of purified recombinant human β-cardiac S1 and cartoon motor construct. Lane 1 contains Invitrogen BenchMark Ladder, and lane 2 contains WT β-cardiac S1. Myosin S1 (residues 1–808) connected by a GAPGSG linker with a C-terminal eGFP runs around 120 kDa and is copurified with a FLAG-tagged human ventricular ELC containing a TEV-precision protease site (24 kDa).
Fig. S2. Single-molecule dwell time and step size measurements. (A) A representative time trace of human β-cardiac WT S1 binding events at 2 mM ATP. Unidirectional (positive) mean bead displacement events were observed upon binding and stroking of the motor. A red line is shown to illustrate four selected events in this trace. (B) A representative histogram of the dwell times from a single WT myosin molecule at 2 mM ATP is shown. Data were fit (solid line) to a maximum likelihood estimation with an exponential function containing a cutoff to determine a mean dwell time (SI Materials and Methods). In total, molecules from more than two motor preparations were examined, with $n = 6$ molecules for WT and $n = 8$ for R453C. (C) A representative histogram of a stroke size distribution from a single WT myosin molecule at 0.5 μM ATP. A Gaussian was fit (solid line) by least squares to the histogram to give a mean stroke size. Molecules from more than two motor preparations were examined, with $n = 14$ molecules for WT and $n = 18$ for R453C. Values reported in the text for both dwell time and stroke size are mean ± SEM.

Fig. S3. Single-molecule intrinsic force measurements with the optical trap. (A) A representative time trace of human β-cardiac WT S1 force events at 0.5 μM ATP. (B and C) Histograms of all events from more than five protein preparations, with (B) $n = 10$ molecules for WT and (C) $n = 14$ molecules for R453C. Each molecule was independently fit for final force measurements. See SI Materials and Methods for further discussion. Values reported in the text for intrinsic force are mean ± SEM.
Fig. S4. ATPase with and without gelsolin at 30 °C. Actin-activated ATPase for WT and R453C S1 with (filled circles) and without gelsolin (empty circles) at a ratio of 1:1,000 were compared. WT is shown in black and R453C data in red. Gelsolin aided in decreasing actin viscosity and increasing the accuracy at higher actin concentrations. Data represent the mean ± SEM (n = 2).

Table S1. Comparison of 23 °C and 30 °C actin-activated ATPase and velocity data for wild-type and R453C mutant β-cardiac S1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Wild type</th>
<th>R453C</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{cat}$ at 23 °C</td>
<td>$7.4 ± 0.4 \text{s}^{-1} (n = 4)$</td>
<td>$5.0 ± 0.2 \text{s}^{-1} (n = 6)$</td>
</tr>
<tr>
<td>$K_m$ at 23 °C</td>
<td>$38 ± 4 \text{μM} (n = 4)$</td>
<td>$28 ± 4 \text{μM} (n = 6)$</td>
</tr>
<tr>
<td>$k_{cat}$ at 30 °C</td>
<td>$16 ± 1 \text{s}^{-1} (n = 5)$</td>
<td>$13 ± 1 \text{s}^{-1} (n = 5)$</td>
</tr>
<tr>
<td>$K_m$ at 30 °C</td>
<td>$31 ± 4 \text{μM} (n = 5)$</td>
<td>$38 ± 4 \text{μM} (n = 5)$</td>
</tr>
<tr>
<td>$Q_{10}$ of $k_{cat}$</td>
<td>$3.1 ± 0.3$</td>
<td>$4.0 ± 0.3$</td>
</tr>
<tr>
<td>Average $v_0$ at 23 °C</td>
<td>$800 ± 40 \text{nm} \cdot \text{s}^{-1} (n = 3)^*$</td>
<td>$610 ± 30 \text{nm} \cdot \text{s}^{-1} (n = 4)^\dagger$</td>
</tr>
<tr>
<td>Average $v_0$ at 30 °C</td>
<td>$1,100 ± 80 \text{nm} \cdot \text{s}^{-1} (n = 4)^*$</td>
<td>$900 ± 80 \text{nm} \cdot \text{s}^{-1} (n = 2)^\dagger$</td>
</tr>
<tr>
<td>$Q_{10}$ of $v_0$</td>
<td>$1.5 ± 0.2$</td>
<td>$1.8 ± 0.3$</td>
</tr>
</tbody>
</table>

Means ± SEM. n, number of experiments for ATPase curves and number of motor preparations for in vitro motility velocities.
*3,688 tracks at 23 °C; 5,535 tracks at 30 °C.
†2,297 tracks at 23 °C; 2,209 tracks at 30 °C.