Single-Molecule Mechanics of R403Q Cardiac Myosin Isolated From the Mouse Model of Familial Hypertrophic Cardiomyopathy


Abstract—Familial hypertrophic cardiomyopathy (FHC) is an inherited cardiac disease that frequently results in sudden death in the absence of any overt symptoms. Many of the cases documented to date have been linked with missense mutations in the β-myosin heavy chain gene. Here we present data detailing the functional impact of one of the most deadly mutations, R403Q, on myosin motor function. Experiments were performed on whole cardiac myosin purified from a mouse model of FHC to eliminate potential uncertainties associated with protein expression systems. The R403Q mutant myosin demonstrated 2.3-fold higher actin-activated ATPase activity, 2.2-fold greater average force generation, and 1.6-fold faster actin filament sliding in the motility assay. The force- and displacement-generating capacities of both the normal and mutant myosin were also characterized at the single molecule level in the laser trap assay. Both control and mutant generated similar unitary forces (∼1 pN) and displacements (∼7 nm) without any differences in event durations. On the basis of the distribution of mean unitary displacements, this mutation may possibly perturb the mechanical coordination between the 2 heads of cardiac myosin. Any of these observations could, alone or possibly in combination, result in abnormal power output and potentially a stimulus for the hypertrophic response. (Circ Res. 2000;86:737-744.)

Key Words: familial hypertrophic cardiomyopathy ■ cardiac myosin ■ R403Q mouse model ■ laser trap ■ molecular motor

Familial hypertrophic cardiomyopathy (FHC) is an inherited cardiac disease that frequently results in the sudden death of young and otherwise healthy individuals.1,2 Although the clinical manifestation of FHC is widely varied, common features include asymmetric septal hypertrophy, potential outflow tract obstruction, myocyte disarray, interstitial fibrosis, and arrhythmia.1-3 Approximately 70% of all FHC cases documented to date have been linked with single point mutations in various contractile proteins of the cardiac sarcomere. These include β-myosin heavy chain (MHC), myosin regulatory light chain, myosin essential light chain, troponin-T, troponin-I, α-tropomyosin, and myosin binding protein-C.3-9 Although a large portion of these point mutations have been localized to the β-MHC gene (n>50 specifically in the motor domain or “head” region),10 few have been characterized in terms of their effect on myosin motor function to the extent of the R403Q substitution. This mutation causes malignant disease, with 50% of the affected individuals dying by 40 years of age.6-11

Investigators seeking to characterize the effects of R403Q have worked with muscle fibers from afflicted individuals, myosin purified from human patients, and genetically engineered fragments of myosin. Because β-MHC is expressed in slow skeletal muscle fibers in addition to adult cardiac tissue,12 Lankford et al13 were able to isolate fibers from the soleus muscles of R403Q FHC patients for mechanical characterization. These fibers exhibited a depressed mechanical state including decreased isometric force generation and lower shortening velocities, resulting in decreased power output and depressed force/stiffness ratios. Is this altered performance due to the inability of R403Q myosin to assemble properly within the sarcomere, or is the myosin motor itself functionally compromised? Recent evidence suggests that in both human fibers and cultured cells, myosin carrying this amino acid change assembles into functional sarcomeric units.14,15 However, Cuda et al16 showed that slow skeletal muscle myosin purified from patients expressing the R403Q mutation produced markedly decreased sliding filament velocities in the in vitro motility assay, suggesting that the motor itself is mechanically compromised.

To obtain larger quantities of purified R403Q MHC for biochemical and mechanical characterization, various laboratories have used protein expression systems to produce myosin fragments presenting this mutation.17-20 Interestingly,
403 substitutions expressed in Dictyostelium myosin II, rat α-cardiac, and human β-cardiac MHCs result in heavy meromyosins that generate slower sliding velocities in the in vitro motility assay, depressed actin-activated ATPase rates, and elevated $K_{m}$ for actin.17–20 Although the in vitro motility and ATPase assays provide useful information concerning the performance of myosins, they are “ensemble” measurements based on large populations of protein. As such, they do not provide information regarding the behavior of these motors at the level of a single molecule.

To understand how the R403Q mutation perturbs myosin function at the molecular level, we performed a single-molecule mechanical assay on native cardiac myosin isolated from homozygous mice expressing only the altered protein.21 Using the mouse model provided an opportunity to examine myosin from animals with a well-characterized phenotype, similar to the disease state experienced by humans (myocyte hypertrophy, slowed relaxation rates, elevated rates of pressure development, interstitial fibrosis, and myofibrillar disarray).21,22 Here we present evidence that the R403Q substitution enhances both hydrolytic and motor function of the mutant cardiac myosin. Furthermore, our interpretation of the data from single-molecule experiments indicates that this mutation may uncouple the mechanical coordination between the 2 “heads” of a cardiac myosin molecule. The results from these single-molecule and ensemble assays together uncover the fundamental perturbations associated with R403Q as well as provide insight into the mechanism of chemomechanical energy transduction in cardiac muscle myosins.

Materials and Methods

FHC Mouse Model

Mice expressing the R403Q mutation were created using recombinant techniques, as previously described,23,24 and expressed ~100% V$_{1}$-myosin (ie, αα-MHC homodimer) 1 week after birth. Control (+/+), heterozygote (+/403), and homozygote (403/403) mice were bred. 403/403 animals lived only 1 week and thus were generally euthanized within this time and compared with age-matched controls. Mice were treated in accordance with the guidelines of the Animal Care and Use Committee of Harvard University.

Protein Purification and Storage

To extract myosin, hearts were homogenized in high-salt buffer (1:5 wt:vol, 0.3 mol/L KCl, 0.15 mol/L KH$_{2}$PO$_{4}$, 0.01 mol/L MgCl$_{2}$, and 0.002 mol/L DTT, pH 6.8) for 20 minutes.25 The homogenate was cleared of cellular debris by ultracentrifugation (60 minutes, 150 000 $g$, 4°C, with Beckman SW41-Ti). The pellet was resuspended in myosin buffer (in mol/L, imidazole 0.025, MgCl$_{2}$ 0.004, DTT 0.01, EGTA 0.001, and KCl 0.3, at pH 7.4) and stored in 50% glycerol. Using the mouse model provided an opportunity to examine myosin from animals with a well-characterized phenotype, similar to the disease state experienced by humans (myocyte hypertrophy, slowed relaxation rates, elevated rates of pressure development, interstitial fibrosis, and myofibrillar disarray).21,22 Here we present evidence that the R403Q substitution enhances both hydrolytic and motor function of the mutant cardiac myosin. Furthermore, our interpretation of the data from single-molecule experiments indicates that this mutation may uncouple the mechanical coordination between the 2 “heads” of a cardiac myosin molecule. The results from these single-molecule and ensemble assays together uncover the fundamental perturbations associated with R403Q as well as provide insight into the mechanism of chemomechanical energy transduction in cardiac muscle myosins.

ATPase Assays

High-salt Ca$^{2+}$- and NH$_{4}$$^{+}$-ATPase activities were measured in either Ca$^{2+}$ assay buffer (10 mmol/L Tris [pH 8.0], 0.23 mol/L KCl, and 2.5 mmol/L CaCl$_{2}$) or NH$_{4}^{+}$ assay buffer (0.4 mol/L NH$_{4}$Cl, 2 mmol/L EDTA, 25 mmol/L Tris [pH 8.0], 0.2 mol/L sucrose, 1 mmol/L DTT, and 1 mg/mL BSA) at 25°C. Actin-activated ATPase activity was measured at multiple actin concentrations (5 to 100 μmol/L) in actin buffer at 20°C (pH 7.4). ATPase activity was determined using 2 mmol/L MgATP (~1.8 mmol/L free Mg$^{2+}$). Inorganic phosphate ($P_{i}$) concentrations at fixed time points were determined colorimetrically, using a malachite green phosphate indicator.27 For the actin-activated assay, values of $P_{i}$ released s$^{-1}$×head$^{-1}$ versus [actin] were plotted and fitted to Michaelis-Menten kinetics ($V = V_{max}×[actin]/K_{m}$×[actin]), with $V_{max}$ and $K_{m}$ fit parameters using Tablecurve 2D version 4 from SPSS.

Motility Assays

Actin filament velocities ($v_{max}$) were measured in low-salt actin buffer (in mol/L, Tris 0.025, MgCl$_{2}$ 0.004, DTT 0.01, EGTA 0.001, and KCl 0.025, at pH 7.4) for each cardiac myosin (+/+, +/403, and 403/403), as previously described.26,28 The relative average isotropic force ($F_{rel}$) of myosin was measured in a “mixture assay” as previously described26 (see Results for description). Details of the optical trap instrumentation and experimental procedures for the single molecule assay have been published elsewhere.29–31 This assay provides estimates of the unitary displacements ($d$) and forces ($F$) of myosin. The estimates of $d$, $F$, and event durations ($t_{ev}$) were obtained by mean-variance (MV) analysis.30 The assay was performed in 1 μmol/L MgATP actin buffer.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

Protein Purification

Whole cardiac myosin was successfully purified from hearts excised from 1-week-old neonatal mice using standard high-salt extraction procedures as outlined in Materials and Methods. PAGE analysis of freshly purified samples (see Figure 1) revealed no differences in purity between +/+ and 403/403 myosins. Moreover, contaminating proteins and proteolysis...
were minimal and thus unlikely to account for any phenotypic differences observed in the various functional assays.

ATPase Activity

Actin-activated ATPase activity of all 3 cardiac myosin species was assessed at multiple actin concentrations (see Materials and Methods). Both 403/403 and +/403 myosins hydrolyzed ATP at rates 2.3 and 1.8 times faster, respectively, than control (Figure 2, Table 1). These results were somewhat unexpected, as all previous studies have shown the rates obtained during these experiments. In these experiments, each “fast” cardiac myosin (+/+ or 403/403) was mixed with different proportions of a “slow” reference myosin (ie, chicken gizzard smooth muscle myosin, for which \( v_{actin} = 1.6 \) \( \mu \text{m/s} \)). By analyzing how the cardiac myosin–based actin filament velocity slowed with the addition of the slower smooth muscle myosin (see Figure 3), an estimate of the relative \( F_{avg} \) for each cardiac myosin was obtained. The observed relationships between the fraction of fast myosin versus sliding velocity were fitted to a model (solid lines, Figure 3) based on the mechanical interaction of 2 myosins having independent force-velocity relationships. In the context of this model, a straight-line fit indicates that the 2 myosin species generate equal force (dashed line, Figure 3). The relationships shown in Figure 3 were concave down (ie, extended below the dashed line) for both the +/+ and 403/403:smooth muscle myosin mixtures, suggesting that smooth muscle myosin generates 3.3 and 1.5 times greater

**Figure 2.** Actin-activated ATPase measurements for +/+ (□), +/403 (△), and 403/403 (□) mouse cardiac myosin. Each data point represents mean±SEM at a given actin concentration. Data were fitted to a Michaelis-Menten kinetic model, \( V_{max} \) and \( K_m \) of the fit parameters are indicated in each case. Three 403/403, two +/403, and four +/+ hearts were used to generate these data. *P<0.05 vs +/+.

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**In Vitro Motility Assay**

The in vitro motility assay measures the ability of a population of myosin motors to propel actin under unloaded conditions. As with the ATPase data, the 403/403 and +/403 myosins demonstrated 60% and 16% higher \( v_{actin} \) respectively, when compared with +/+ myosin (see Table 1). Previous investigations of the R403Q mutation in other myosin systems have reported a decline in filament motility to as little as 20% of control values. Motility experiments contributing to the data in Table 1 were performed on numerous hearts from multiple litters, with the 403 mutants demonstrating consistently higher velocities.

In the first week after birth, a rapid myosin isoform shift occurs within the mouse heart from being predominantly \( \beta \)-MHC to being predominantly \( \alpha \)-MHC. Because the hearts used in this work were obtained during this period, altered cardiac MHC content could contribute to the observed functional differences. To test this hypothesis, we performed in vitro motility on purified myosin from mouse hearts ranging from 4 to 8 days old. These experiments, in conjunction with SDS-PAGE of corresponding samples, revealed that any isoform shifts were complete by 4 days after birth and that myosins isolated from 4- to 8-day-old hearts were functionally indistinguishable (data not shown).

**Measurement of Relative Isometric Force**

Relative levels of isometric force (\( F_{avg} \)) produced by the +/+ and 403/403 myosins were determined using the in vitro motility “mixture” protocol (Figure 3, Table 1). In these experiments, each “fast” cardiac myosin (+/+ or 403/403) was mixed with different proportions of a “slow” reference myosin (ie, chicken gizzard smooth muscle myosin, for which \( v_{actin} = 1.6 \) \( \mu \text{m/s} \)). By analyzing how the cardiac myosin–based actin filament velocity slowed with the addition of the slower smooth muscle myosin (see Figure 3), an estimate of the relative \( F_{avg} \) for each cardiac myosin was obtained. The observed relationships between the fraction of fast myosin versus sliding velocity were fitted to a model (solid lines, Figure 3) based on the mechanical interaction of 2 myosins having independent force-velocity relationships. In the context of this model, a straight-line fit indicates that the 2 myosin species generate equal force (dashed line, Figure 3). The relationships shown in Figure 3 were concave down (ie, extended below the dashed line) for both the +/+ and 403/403:smooth muscle myosin mixtures, suggesting that smooth muscle myosin generates 3.3 and 1.5 times greater

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**TABLE 1. Summary of Ensemble Measurements**

<table>
<thead>
<tr>
<th>Myosin</th>
<th>( NH_4^-\text{-ATPase, s}^{-1}\times\text{head}^{-1} )</th>
<th>( Ca^{2+}\text{-ATPase, s}^{-1}\times\text{head}^{-1} )</th>
<th>Actin-Activated ( V_{max} ), s(^{-1}\times\text{head}^{-1} )</th>
<th>Actin-Activated ( K_v ), ( \mu\text{mol/L} )</th>
<th>( v_{actin} ), ( \mu\text{m/s} )</th>
<th>( F_{avg} ) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>15.2±1.1 (2)</td>
<td>0.26±0.06 (2)</td>
<td>1.6±0.2 (4)</td>
<td>5.5±2.4 (4)</td>
<td>5.5±1.1 (13)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>+/403</td>
<td>14.2±1.1 (2)</td>
<td>0.19±0.01 (2)</td>
<td>2.9±0.6 (2)</td>
<td>24.1±11.6 (2)</td>
<td>6.4±1.2 (3)</td>
<td>...</td>
</tr>
<tr>
<td>403/403</td>
<td>13.6±0.5 (2)</td>
<td>0.15±0.04 (2)</td>
<td>3.6±0.6* (3)</td>
<td>19.4±2.3 (3)</td>
<td>9.0±1.9* (9)</td>
<td>2.2* (3)</td>
</tr>
</tbody>
</table>

*Means are given in each column, ±SE of the Michaelis-Menten fit for \( V_{max} \) and \( K_v \), and ±SD for the high-salt ATPase rates and \( v_{actin} \). Numbers in parentheses indicate number of hearts contributing to the parameter listed.

*In the case of \( F_{avg} \), the parameter listed is the inverse of the ratio derived from the fit to the mechanical interaction model (see Figure 3) normalized to the control value.

†P<0.05 vs +/+. 

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average force compared with the +/+ and 403/403 cardiac myosins, respectively. The enhanced force-generating capacity of smooth muscle myosin relative to either V₁ or V₃ rabbit cardiac myosin has been reported previously. 32 Using smooth muscle myosin as a common reference, it appears that the 403/403 myosin produces higher force (P_avg) when compared with purified +/+ myosin. Three 403/403 and two +/+ hearts were used to generate these data. *P<0.05 vs +/+. 

**Unitary Mechanical Measurements**

To understand the enhanced force and motion-generating ability of 403/403 myosin at the molecular level, we measured the mechanics of single cardiac myosin molecules in the optical trap (see Materials and Methods). 30 For these experiments, only preparations providing homogenous populations of MHC were assayed (ie, +/+ and 403/403). Representative unitary displacement and force records are shown in Figure 4. Both displacement and force events appeared as rapid deflections from baseline (indicated by arrowheads in Figure 4). MV analysis (see Materials and Methods) was used to estimate d, F, and average event durations (tₑ) from the time series data. Under the lightly loaded conditions of the optical trap (ie, low trap stiffness), d values produced by the +/+ and 403/403 myosins were comparable at ~7 nm (see Table 2). Likewise, under high load, F values produced by +/+ and 403/403 myosins were also very similar at ~1 pN (see Table 2). Interestingly, +/+ and 403/403 myosins produced similar tₑ for displacement (~70 ms) and force events (~175 ms; see Table 2).

The only potential difference between +/+ and 403/403 myosins was found in the distribution of mean displacement event amplitudes, as shown in Figure 5 (see also dₐ in Table 2). Each data point represents the mean event amplitude estimated by MV analysis of a displacement time series record containing tens to hundreds of events generated by a single myosin molecule. Therefore, the entire distribution represents behavior of multiple independent myosin molecules. In this plot, the +/+ distribution was best fit by 2 gaussian curves with individual peaks at 5.2 and 9.2 nm (see Figure 5 legend). In contrast, the 403/403 distribution was well fit by a single peak at 6.3 nm (see Figure 5 legend).

**Discussion**

The ensemble measurements presented here reveal that the R403Q mutation associated with the mouse model of FHC results in a gain of hydrolytic and mechanical function; ie, actin-activated ATPase rates, Vₑ actin, and F_avg were all elevated (Table 1, Figures 2 and 3). Interestingly, the enhanced performance was only a property of assays involving the interaction of actin and myosin, as the intrinsic ATPase rates measured under high-salt conditions were not significantly different from those of controls (see Table 1). This finding can be explained, because the R403 amino acid is thought to be located on the putative actin-binding interface of myosin. 10
Means are given in each column: ± SD. Numbers in parentheses represent the number of MV histograms contributing to given a mean. Here, a single MV histogram may represent up to several hundred events. The same histograms described under d were used to generate the fits given under d*. d* represents means from best gaussian fits to the frequency histograms in Figure 5 (see Figure legends). Six +/- hearts and seven 403/403 hearts were used to obtain these data. \( \tau_\text{m} \) was measured from force and displacement records at 1 \( \mu \text{mol/L} \text{MgATP} \).

Therefore, only measurements involving the formation of the actomyosin complex should be perturbed. Indeed, this result has also been shown by others.\(^{17} \)

Is this enhanced functional state unique to the in vitro assays performed here? In situ functional measurements conducted in +/- mouse hearts indicate that these muscles are indeed hypercontractile and display a significantly elevated \( +\text{dP/dt} \).\(^{22} \) This finding is consistent with the faster velocities and higher forces reported here for the 403 mutation. Blanchard et al.\(^{33} \) studying papillary muscles from +/- mouse hearts, also observed elevated isometric tension at submaximal activation. However, isometric tension was equal to controls at maximal activation, a result that may be related to the fiber having a contractile system with intact regulatory proteins. The in vitro experiments reported here were all performed using unregulated filament actin.

This report is the first to document a “gain of function” resulting from the R403Q substitution. This is in direct contrast to earlier reports from other groups.\(^{17–20} \) One possible explanation for this discrepancy may relate specifically to using the mouse as a model system. For example, the functional impact of this mutation may vary when expressed in MHC backbones from different species. However, the enhanced motor function may not be unique to mouse cardiac myosin, as preliminary data from chicken gizzard smooth muscle myosin expressing the R403Q mutation suggest that actin-activated ATPase and \( v_\text{act} \) are accelerated in this case as well.\(^{34} \) As the majority of previous studies have been performed on expressed myosin fragments (eg, from the baculovirus system), it is worth noting that investigators have had an exceedingly difficult time trying to express striated muscle myosin fragments at high yield.\(^{17} \) This leads to the possibility that the myosin fragments expressed with the 403 mutation in these in vitro systems may be compromised for reasons other than the single amino acid substitution at position 403. The fact that native whole cardiac myosin purified for this study actually demonstrated enhanced performance highlights this possibility. It should also be noted that in the human disease state, the 403 mutation is found in V\(_1\)-cardiac myosin (ie, \( \beta\beta\)-MHC homodimer), the isoform predominantly expressed in adults. In contrast, our mouse hearts are \( \approx 100\% \) V\(_1\)-myosin (ie, \( \alpha\alpha\)-MHC homodimer). Although primary sequences of the \( \alpha\)- and \( \beta\)-MHCs are remarkably similar, minor variations in functionally significant regions\(^{35} \) are thought to contribute to the different characteristics of the 2 isoforms.\(^{36} \) Therefore, it is conceivable that the same mutation presented in a \( \beta\)-MHC might have distinct consequences. However, we have also demonstrated similar enhanced function in myosin isolated from cardiac biopsy samples from human FHC patients.\(^{37} \)

In humans, the FHC disease state is a heterozygous condition, where only 1 allele contains the mutation. Given that myosin consists of 2 heavy chains and that the expression of the normal and mutant heavy chain appears to be equal,\(^{12} \)

### Table 2. Summary of Unitary Mechanical Data

<table>
<thead>
<tr>
<th>Myosin</th>
<th>( F_i ), pN</th>
<th>( \tau_\text{m} ), ms</th>
<th>d, nm</th>
<th>( \tau_\text{m} ), ms</th>
<th>( d^* ), nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/-</td>
<td>0.9±0.4 (15)</td>
<td>174.0±72.7 (14)</td>
<td>7.1±2.3 (57)</td>
<td>67.9±20.4 (20)</td>
<td>5.2±1.0/9.2±1.3</td>
</tr>
<tr>
<td>403/403</td>
<td>1.3±0.5 (7)</td>
<td>177.1±56.5 (6)</td>
<td>6.5±2.3 (34)</td>
<td>73.2±21.4 (37)</td>
<td>6.3±2.4</td>
</tr>
</tbody>
</table>

Figure 5. Scatter plots representing the distributions of MV histogram displacement fits from +/- (left) and 403/403 (right) mouse cardiac myosin. In these representations, each open circle indicates the mean value from a single MV histogram fit generated from 1 displacement record (+/+, n=57 records; 403/403, n=34 records). Therefore, each circle entered into these plots represents 50 to 100+ unitary events. Six +/- hearts and seven 403/403 hearts were used to obtain these data. Bar histograms below each scatter plot are frequency distributions for the same data. In each case, data were fit with both single and double gaussian functions. Best fit (displayed on each plot) was chosen as the fit that produced the highest \( F \) statistic. Parameters from the fits were as follows: +/- first peak amplitude, 11.8 (mean, 5.2; SD, 1.24); second peak amplitude, 9.8 (mean, 9.2; SD, 1.3); and 403/403 amplitude, 8.17 (mean, 6.3; SD, 2.6). Means are also indicated above each gaussian peak in the figure.
then one would expect myosin to assemble in vivo as a mixture containing 25% +/- homodimers, 50% +/-/403 heterodimers, and 25% 403/403 homodimers. The enhanced function demonstrated in the ATPase and in vitro motility assays was clearly evident, not only for the 403/403 homodimer, but also for myosin from the +/-/403 heterozygote. If only the 403/403 homodimers contributed to the observed increase in ATPase activity for myosin from the heterozygote mouse, then only a 31% increase would have been predicted as compared with the 80% observed. This suggests that only 1 of the MHCs within a molecule needs to possess the R403Q substitution in order for the enzymatic and mechanical phenotypes to be significantly altered. Given that 75% of the myosin molecules in the human condition will contain at least 1 mutant MHC, it is not surprising that the heterozygous individual will present the clinical manifestations of the disease.

On the basis of the enhanced \( v_{\text{actin}} \) and \( F_{\text{avg}} \) observed for a population of 403/403 myosin motors, is it possible to understand the molecular mechanism by which this mutation exerts its effects? At the single-molecule level,

\[
v_{\text{actin}} = \frac{d}{t_{\text{on}}}
\]

where \( d \) is the unitary displacement and \( t_{\text{on}} \) is the displacement duration. In addition,

\[
F_{\text{avg}} = F \times f_{\text{iso}}
\]

where \( F \) represents the unitary force developed by a single molecule and \( f_{\text{iso}} \) is the isometric “duty cycle” or fraction of the total cycle time (\( t_{\text{cycle}} \)) that the motor spends strongly bound to actin exerting \( F \), written as

\[
f_{\text{iso}} = t_{\text{on}}/t_{\text{cycle}}
\]

Assuming these molecular definitions for \( v_{\text{actin}} \) and \( F_{\text{avg}} \), one can then determine whether the mutation has its effect by changing either the inherent mechanical (ie, a change in \( d \) or \( F \)) and/or kinetic (ie, a change in \( t_{\text{on}} \) or \( f_{\text{iso}} \)) properties of myosin. Given the results of unitary mechanical measurements, we believe that both \( F \) and \( d \) produced by the 403/403 myosin were similar to that of the +/- myosin. If so, then based on Equations 1 and 2, in the absence of any change in mechanical properties, the enhanced average force and velocity should be due to a change in the cycling kinetics of the myosin molecule. However, there were no apparent differences in the \( t_{\text{on}} \) estimated for both unitary displacements and forces for the 403/403 and +/- myosins.

Perhaps kinetic differences do exist between +/- and 403/403 myosin but were not resolved under the 1 \( \mu \text{mL} \) MgATP conditions of the optical trap assay. This potential difficulty stems from the fact that there are 2 crossbridge cycle transitions that contribute to the event durations: MgADP release from myosin and the subsequent rebinding of MgATP.\(^{36,38}\) Under the 1 \( \mu \text{mL} \) MgATP conditions of the in vitro motility assay, the ATP rebinding rate is exceedingly high, and thus MgADP release governs the attached duration (ie, \( t_{\text{on}} \)), in turn limiting \( v_{\text{actin}} \).\(^{36,39-41}\) At 1 \( \mu \text{mL} \) MgATP, the MgATP rebinding rate is several times slower than ADP release,\(^{36,41}\) raising the possibility that a difference in MgADP release rate between +/- and 403/403 could exist but would be obscured by the low-MgATP assay conditions. This possibility may explain why, under both loaded and unloaded conditions, there were no measurable differences in the \( t_{\text{on}} \) values for +/- and 403/403 myosins (see Table 2). Unfortunately, because of the rapid kinetics of the \( \alpha \)-MHC,\(^{36}\) performing experiments at 1 \( \mu \text{mL} \) MgATP in an effort to resolve this issue would produce \( t_{\text{on}} \) values comparable with the temporal resolution of our instrument (\(~2\) to 5 ms).

Despite the lack of a difference in \( t_{\text{on}} \) from unitary force measurements, a kinetic change can still explain why \( F_{\text{avg}} \) was elevated in the case of the mutant. Assuming that ATPase differences measured in solution (see Table 1) reveal information about cycling kinetics under load as well, the increased ATPase rate measured for 403/403 myosin may help to explain why \( F_{\text{avg}} \) was higher. Because \( t_{\text{cycle}} = (\text{ATPase rate})^{-1} \), a higher ATPase activity means a shorter overall cycle time, which, in the absence of a change in \( t_{\text{on}} \), would result in an increase in \( f_{\text{iso}} \) (see Equation 3). As indicated by Equation 2, an increase in \( f_{\text{iso}} \) can explain the higher \( F_{\text{avg}} \) produced by 403/403 myosin.

Interestingly, another potential functional difference between 403/403 and +/- reveals itself on closer inspection of the single-molecule data. The scatter plots shown in Figure 5 demonstrate that the mutation has a profound effect on the distributions of displacement amplitudes. In this representation, the +/- distribution of displacements is better fit by a bimodal distribution, whereas the 403/403 scatter plot is better described by a single gaussian (see legend to Figure 5). Using a similar scatter plot analysis, Tyska et al\(^{42}\) recently described a molecular-level comparison of single- and double-headed myosins. These data indicate that double-headed muscle myosins coordinate their action to produce twice the force and motion in the optical trap assay when compared with their single-headed counterparts. Given this evidence, we propose that the bimodal distribution shown for +/- myosin arises from the functional relationship between the 2 heads of cardiac myosin. It is likely that the bimodal distribution shown for +/- myosin represents the action of both heads (peak at 9.2 nm, Figure 5) or, at times, a single cardiac myosin head (peak at 5.2 nm, Figure 5). The shift in mass toward the lower distribution may indicate that 403/403 myosin is behaving more “single-headed” than the +/- control, with a single peak at 6.3 nm (Figure 5). This is consistent with the mean value we have reported for single-headed smooth and skeletal muscle myosins.\(^{43}\) At present, the precise mechanism linking potential changes in head-head coordination to observed changes in ATPase activity, \( v_{\text{actin}} \) or \( F_{\text{avg}} \), is unclear and requires further investigation.

How could the enhanced function observed in the in vitro assays result in a disease state in vivo? One potential explanation may relate to the abnormally high levels of energy consumption associated with the increased ATPase activity for the 403/403 mutant. This is consistent with the nuclear magnetic resonance spectroscopic data of Spindler et al,\(^{43}\) demonstrating perturbations in the levels of high-energy phosphate compounds in +/- myosin mouse hearts. The authors reasoned that this would ultimately result in a decreased free energy for ATP hydrolysis within the cell,\(^{43}\) and, because
other cellular ATPases have high free energy demands (eg, sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) ATPase pump), they run the risk of becoming “energy starved.” This energetic imbalance could be one possible stimulus leading to cardiac hypertrophy. A second possibility relates to the higher force levels produced by the 403 mutant myosin. If the cardiac sarcomere is designed to function with tolerance for a normal range of physiological force development, then abnormally high levels of average force could be the origin of sarcomeric and myocyte disarray seen in FHC-affected hearts.\(^{21}\)

The in vitro functional analysis presented here has revealed that the R403Q mutation present in the mouse model of FHC may exert its effect through a combination of kinetic perturbations that alter ensemble force (\(F_{\text{avg}}\)), velocity (\(v_{\text{max}}\)), and ATPase (\(V_{\text{max}}\)) and possibly the suppression of the native level of head-head coordination in mouse V\(_1\)-cardiac myosin. Through these effects, the R403Q mutation creates a stronger, faster myosin so that under any load, the power output of these hearts should be augmented beyond the tolerance of a normal cardiac sarcomere. The in vivo significance of the functional alterations reported here is highlighted by the fact that these effects are likely the fundamental stimuli for the hypertrophic response in the R403Q mouse model of FHC.

Acknowledgments

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References


Single-Molecule Mechanics of R403Q Cardiac Myosin Isolated From the Mouse Model of Familial Hypertrophic Cardiomyopathy

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