Transgenic Mouse \(\alpha\)- and \(\beta\)-Cardiac Myosins Containing the R403Q Mutation Show Isoform-dependent Transient Kinetic Differences*

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Background: The mouse model for cardiac disease has been the focus for mutational studies on the \(\alpha\)-cardiac myosin heavy chain (MHC).

Results: Transient kinetics showed that R403Q enhanced ADP release in mouse \(\alpha\)-MHC but not in mouse \(\beta\)-MHC.

Conclusion: The functional impact of a mutation depends on the myosin isoform.

Significance: The R403Q mutation in human \(\beta\)-cardiac myosin likely compromises its function leading to hypertrophic cardiomyopathy.

Familial hypertrophic cardiomyopathy (FHC) is a major cause of sudden cardiac death in young athletes. The discovery in 1990 that a point mutation at residue 403 (R403Q) in the \(\beta\)-myosin heavy chain (MHC) caused a severe form of FHC was the first of many demonstrations linking FHC to mutations in muscle proteins. A mouse model for FHC has been widely used to study the mechanoochemical properties of mutated cardiac myosin, but mouse hearts express \(\alpha\)-MHC, whereas the ventricles of larger mammals express predominantly \(\beta\)-MHC. To address the role of the isoform backbone on function, we generated a transgenic mouse in which the endogenous \(\alpha\)-MHC was partially replaced with transgenically encoded \(\beta\)-MHC or \(\alpha\)-MHC. A His\(_6\) tag was cloned at the N terminus, along with R403Q, to facilitate isolation of myosin subfragment 1 (S1). Stopped flow kinetics were used to measure the equilibrium constants and rates of nucleotide binding and release for the mouse S1 isoforms bound to actin. For the wild-type isoforms, we found that the affinity of MgADP for \(\alpha\)-S1 (100 \(\mu\)M) is \(\approx\) 4-fold weaker than for \(\beta\)-S1 (25 \(\mu\)M). Correspondingly, the MgADP release rate for \(\alpha\)-S1 (350 s\(^{-1}\)) is \(\approx\) 3-fold greater than for \(\beta\)-S1 (120 s\(^{-1}\)). Introducing the R403Q mutation caused only a minor reduction in kinetics for \(\beta\)-S1, but R403Q in \(\alpha\)-S1 caused the ADP release rate to increase by 20% (430 s\(^{-1}\)). These transient kinetic studies on mouse cardiac myosins provide strong evidence that the functional impact of an FHC mutation on myosin depends on the isoform backbone.

Familial hypertrophic cardiomyopathy (FHC)\(^2\) is a disease of the heart whose characteristic features include left ventricular hypertrophy, increased fibrosis and myocyte disarray. A genetic basis for this disease was first shown by the Seidmans in 1990 (1) with the discovery that a single missense mutation, R403Q, in the human \(\beta\)-myosin heavy chain (MHC) was responsible for a particularly lethal form of FHC. Since that time several hundred mutations in genes encoding sarcomeric proteins have been identified, with the largest number (>300) located in the \(\beta\)-MHC. In the first decade of research following the discovery of R403Q, studies of this mutation in species as diverse as the soil amoeba (Dictyostelium discoideum) and humans all showed a “loss of function” in actomyosin interactions (reviewed in Ref. 2). However, with the introduction of the mouse model for R403Q in 1996 (3), evidence shifted to a “gain of function” caused by this heavy chain mutation (4).

All mammalian species contain two cardiac myosin heavy chain isoforms that are encoded by two \(\alpha\)- and \(\beta\)-MHC genes in tandem: \(\alpha\)-MHC is expressed in the atrium, and \(\beta\)-MHC is the predominant cardiac myosin isoform in the ventricles (5). In a recent publication, we sought to answer the question of whether the “gain of function” found for mice containing the R403Q mutation was linked to their \(\alpha\)-MHC backbone (6). By means of transgenesis (TG), it was possible to partially replace the endogenous \(\alpha\)-MHC in the adult mouse with expressed \(\beta\)-MHC (7). To facilitate protein isolation, a His\(_6\) tag was cloned at the N terminus of both \(\alpha\)- and \(\beta\)-MHC in the absence (wild type) or presence of the R403Q mutation. A homogeneous population of myosin subfragment 1 (S1) could then be prepared by digesting the myosin with chymotrypsin followed by Ni\(^{2+}\) chelating chromatography to isolate the His\(_6\)-tagged S1. Steady-state actin-activated MgATPase measurements showed that enzymatic activity is enhanced \(\sim\)30% for S1 (R403Q) in an \(\alpha\)-MHC backbone, but R403Q had little effect on S1 activity in a \(\beta\)-MHC backbone. The in vitro motility assay showed enhanced actin filament translocation by R403Q \(\alpha\)-myosin compared with a control, consistent with earlier reports (4).

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2 The abbreviations used are: FHC, familial hypertrophic cardiomyopathy; MHC, myosin heavy chain; S1, subfragment 1, TG, transgenic.
However, the motility assay with R403Q β-myosin was not definitive, because of the unavoidable presence of some endogenous fast cycling α-myosin heads, which compromised interpretation of the data (6).

To gain further insight into the mechanochemical properties of myosin mutants in two isoform backbones, we turned to stopped flow kinetics to measure the rate of ADP release from myosin (S1) heads, $k_{AD}$. It has been shown for a variety of fiber types that there is a good correlation between unloaded shortening velocity ($v$) and ADP release from the myosin cross-bridge, $v = d^{-1}t^{-1}$, where $d$ is the working stroke and $t$ is the lifetime of the strongly attached state (8, 9). Once ADP is released, ATP rebinds to the active site and causes rapid dissociation of the cross-bridge from actin to restart the cycle (see Fig. 1). Thus, determination of $k_{AD}$ by transient kinetics provides a means to estimate the velocity of actin filament movement by S1, which could not be determined directly by in vitro motility measurements. Here we report that the effect of the R403Q mutation on cardiac myosin does indeed depend on the nature of the isoform: the R403Q mutation has a more profound effect of cardiac myosin, with hypertrophy following as a compensatory response for the dysfunctional heart.

**EXPERIMENTAL PROCEDURES**

**Transgenic Animals**—TG mice with cardiac-specific expression of mutant and wild-type His$_{6}$-tagged α- and β-MHC were generated as described by Lowey and Lowey (6). The cardiac myosin subfragments used here were prepared from mouse hearts that had been stored at −80 °C from the earlier experiments.

**Preparation and Purification of S1**—Mouse cardiac myosin was prepared from ~2 g of tissue (20 or more frozen mouse hearts). The details of the preparation are described by Lowey et al. (6). Briefly, the thawed tissue was homogenized in an imidazole buffer and clarified by centrifugation until the supernatant was nearly colorless. The pellet was homogenized in 15 ml of extraction buffer (150 mM sodium phosphate, pH 7.0, 0.3 M NaCl, 10 mM pyrophosphate, 2 mM MgCl$_2$, 1 mM EGTA, 1 mM DTT, and protease inhibitors), and the suspension was stirred for 30 min. After centrifugation, the supernatant was diluted ~12-fold with water containing 0.5 mM DTT. The precipitated protein was centrifuged, and the pellets were dissolved in 0.5 M NaCl, 25 mM sodium phosphate, pH 7.0, 1 mM EGTA, 0.2 mM DTT, 1 µg/ml leupeptin, and dialyzed overnight against the same buffer. This preparation was used as the starting material for the preparation of S1.

Prior to proteolytic digestion, the His$_{6}$-tagged myosin was reacted with 0.3 mM MgATP to dissociate any residual actomyosin and clarified by centrifugation. After overnight dialysis against 20 mM HEPES, pH 7.0, 0.12 M NaCl, 1 mM EDTA, 0.2 mM DTT, and 1 mM NaN$_3$, 1 mg/ml chymotrypsin (dissolved in 1 mM HCl) was added dropwise to the myosin suspension at room temperature to a final concentration of 0.05 mg/ml and stirred for 15 min. The digestion was stopped with 2 mM 4-(2-aminophenyl)-benzenesulfonyl fluoride. The myosin digest was centrifuged to pellet undigested myosin and rod, and the supernatant, containing mainly S1, was loaded onto a 5-ml HiTrap Ni$^{2+}$-charged column (GE Healthcare). Buffer A consisting of 0.5 M NaCl and 20 mM HEPES, pH 7.5, was used to equilibrate the column, and buffer B (same composition as buffer A) had added 0.3 M imidazole for competitive elution. Nonspecifically bound S1 was eluted at 30 mM imidazole (Fluka), and the His$_{6}$-tagged S1 was eluted by stepping the imidazole concentration to 120 mM. The protein was collected in approximately three fractions of 1 ml each and dialyzed versus 55% glycerol buffer containing 20 mM KCl, 20 mM imidazole, pH 7.5, 1 mM EGTA, 1 mM MgCl$_2$, 1 mM NaN$_3$, and 1 mM DTT for storage at −20 °C.

**Preparation of Labeled Actin**—Skeletal muscle actin was prepared from chicken pectoralis acetone powder (10) and stored at 4 °C as F-actin (10–15 mg/ml) in 5 mM KCl, 2 mM MgCl$_2$, 5 mM imidazole, pH 7.5, and 3 mM NaN$_3$. It was used within 2–3 weeks of preparation. For the stopped flow experiments, the actin was labeled with N-(1-pyrenyl)iodoacetamide at cysteine 374 as described by Trybus et al. (11) and Criddle et al. (12). Actin (1 mg/ml in 10 mM imidazole, pH 7.0, 10 mM NaCl, 1 mM MgCl$_2$, 1 mM MgATP) was fully reduced by the addition of 2 mM DTT at pH 8.0 and then dialyzed overnight against 10 mM imidazole, pH 7.8, 0.1 M NaCl, 1 mM MgCl$_2$, and 1 mM NaN$_3$. A 2-fold molar excess of pyrene-iodoacetamide was added from a 4 mM stock in dimethylformamide. After 20 h of labeling by rotation in the dark at 4 °C, the actin was centrifuged, and the pellets were resuspended in 10 mM HEPES, pH 7.0, 0.1 M NaCl, 5 mM MgCl$_2$, 1 mM EGTA, 1 mM DTT, and 1 mM NaN$_3$. Protein concentration was determined by the Bradford assay (Pierce) with bovine serum albumin as the standard.

**Stopped Flow Fluorescence Measurements**—The majority of experiments were done in 10 mM imidazole, pH 7.4, 100 mM NaCl, 1 mM MgCl$_2$, 1 mM DTT, and 1 mM NaN$_3$ at 20 °C, using a KinTek stopped flow model SF-2001 spectrophotometer (dead time, ~1.5 ms) fitted with a xenon/mercury lamp and monochromator. Pyrene was excited at 360 nm, and emission was detected with a 400-nm cut-off filter. All nucleotide stocks were prepared with an equimolar amount of magnesium. The temperature of the syringe chamber, mixer, and observation cell was maintained at 20 °C (with the exception of one experiment at 11 °C) by a circulating water bath. One syringe contained acto S1 with 0–2 mM ADP, whereas the second syringe contained varying concentrations of ATP. The rate of dissociation of acto S1 by ATP in the presence and absence of MgADP was monitored by the increase in pyrene fluorescence with time. Each fluorescence transient was an average of three to eight independent mixings. The signal averaging and fitting was done using KinTek software. The data were well fit by a single exponential equation. Typically, 400 µl of acto S1 was used per data set. Concentrations refer to amounts after 1:1 mixing. The final concentration of S1 was ~0.5 µM with acto to S1 at a 4:1 molar ratio. The $k_{off}$ values were fitted to Equations 1 and 2 to
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FIGURE 1. The basic actomyosin contractile cycle. Myosin (M) is dissociated from actin (A) by ATP, which is rapidly hydrolyzed to ADP, Pi (green). Upon rebinding to actin (orange), the initial weak binding states release phosphate (P) and undergo a lever arm swing (power stroke) to form the strong binding ADP and rigor states. The primary steps in the cycle with the equilibrium constants for ATP ($K_1$) and ADP ($K_{AD}$) binding and the rates for AM dissociation ($k_1$) and product release are indicated in schematic form below the graphic representation.

\[
\text{M-ATP} \rightleftharpoons \text{M-ADP-Pi}
\]

\[
\begin{align*}
\text{A-M} & \quad \text{K}_1 \\
\text{A-M-ATP} & \quad \text{K}_2 \\
\text{A-M-ADP-Pi} & \quad \text{K}_{AD} \\
\text{A-M-ADP} & \quad \text{A-M}
\end{align*}
\]

\[
\begin{align*}
\text{AM} + \text{T} & \quad k_{s2} \\
\text{A-M.T} & \quad \text{K}_{AD} \\
\text{A-M.T} & \quad \text{A} + \text{MT}
\end{align*}
\]

\[\text{AM} + \text{T} \quad k_{s2}
\]

\[\text{A-M.T} \quad \text{K}_{AD}
\]

\[\text{A-M.T} \quad \text{A} + \text{MT}
\]

A.M.D

SCHEME 1. Interactions between S1 and actin with ATP and ADP.

obtain the rate and equilibrium constants described in the contractile cycle in Fig. 1.

Statistical Analysis—Statistical significance ($p$ values) was determined by performing a two-tailed Student’s $t$ test on data obtained from three independent preparations each of TG $\alpha$-MHC (R403Q) and WT $\alpha$-myosin and two preparations each of TG $\beta$-MHC (R403Q) and WT $\beta$-myosin. The figures show a single representative experiment.

Interpretation of Kinetic Data—The data were interpreted in terms of the fundamental interactions between S1 and actin with ATP and ADP (Fig. 1) as shown in Scheme 1. As with most enzymes, there is a saturation of catalytic activity as the substrate concentration is increased. The observed rate constant, $k_{obs}$, for the dissociation of acto S1 (AM) by ATP (T) is given by Equation 1, where $K_1k_{s2}$ is the second order rate constant for the dissociation of the AM complex, and $K_1$ is the equilibrium association constant for AM plus T. At high ATP concentrations, the observed rate constant will be $k_{s2}$ whereas at low ATP, $K_1k_{s2}$ is obtained from the linear dependence of $k_{obs}$ with ATP (Equation 1). The binding of ADP to myosin can be treated as a competitive inhibition reaction. The equilibrium dissociation constant, $K_{AD}$, is obtained from the binding of varying concentrations of ADP (D) to acto S1 (AM) at a constant low concentration of ATP (Equation 2).

\[
k_{obs} = K_1k_{s2}[\text{ATP}]/(1 + K_1[\text{ATP}])
\]

(Eq. 1)

\[
k_{obs} = K_1k_{s2}[\text{ATP}]/(1 + [\text{ADP}]/K_{AD})
\]

(Eq. 2)

RESULTS

Isolation of TG Mouse Cardiac Myosin S1 Isoforms—The isolation and purification of the myosin subfragment 1 (S1) isoforms has been described in detail previously (6). However, because the homogeneity and reproducibility of the protein preparation is so important when analyzing a disease mutation that is expected to cause only a 20–30% change in kinetic parameters, a brief discussion of the procedure is warranted here.

Cardiac myosin is first prepared from 10–20 adult mouse hearts (<100 mg each), the number depending on the level of expression of the mutation in the transgenic mouse. Although His$_6$-tagged wild-type $\beta$-myosin could replace endogenous $\alpha$-myosin by 60–70% with no ill effects, mice expressing the His$_6$-tagged $\beta$-R403Q transgene had poor survival rates, and the amount of mutant $\beta$-myosin replaced did not exceed 10–15%. In contrast, His$_6$-tagged R403Q $\alpha$-myosin could replace up to 50% of the endogenous protein with no harmful effects on the animal. The expression levels of the mutant $\beta$-myosin could be increased somewhat by cross-breeding with a knock-out mouse line as described by Lowey et al. (6), but expression levels were still low compared with mutant $\alpha$-myosin.

Cardiac myosin was prepared from the tissue by standard procedures and digested with chymotrypsin on the second day of preparation as described under “Experimental Procedures.” The crude soluble S1 was applied to an affinity column and eluted at 30 mM imidazole for the untagged fraction and at 120 mM imidazole for the His$_6$-tagged S1. A typical experiment is shown in Fig. 2. After concentrating the peak fractions by dialysis against 55% glycerol, the protein concentration was typically ~0.5–1 mg/ml in a volume of ~1 ml, and the material was stored at −20 °C. This procedure minimized denaturation and ensured a high degree of reproducibility in the subsequent measurements.

Stopped Flow Measurements for Acto $\alpha$-S1 at 11 °C—Anticipating that mouse cardiac myosin isoforms would most likely have fast kinetic properties based on our earlier in vitro motility assays, we initially did experiments at 11 °C following the procedures used by Geeves and co-workers (13) for fast skeletal muscle myosins. We found that the rate of ADP release from acto $\alpha$-S1 ($k_{-AD}$) was comparable to the rate of dissociation of acto $\alpha$-S1 by ATP ($k_{s2}$), and therefore the release of ADP does not appear to limit the velocity of shortening in mouse cardiac myosin to the same extent as in slow myosins of larger mam-
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TABLE 2
Comparison of kinetic constants for α- and β-cardiac S1 from transgenic wild-type and R403Q mouse myosin at 20 °C. The measurements were made in 10 mM imidazole, pH 7.4, 100 mM NaCl, 1 mM MgCl₂, 1 mM DTT, and 1 mM NaN₃.

| Sample | $k_{-S1}$ | $k_{-AD}$ | $K_{AD}$ | $k_{-AD}$ | $k_{AD}$ |
|--------|------------|------------|----------|------------|----------|
| α-S1 (WT) | 1.34 ± 0.01 | 1120 ± 33 | 252 ± 3.4 | 4.96 | 123 ± 13 |
| Exp. 1 | 152 ± 5 | 151 ± 4 |
| Exp. 2 | | |
| β-S1 (R403Q) | 1.64 ± 0.02 | 997 ± 16 | 89 ± 9 | 2.85 | 351 ± 17 |
| Exp. 1 | 342 ± 18 | 375 ± 24 |
| Exp. 2 | | |
| Exp. 3 | | |

FIGURE 4. Fluorescence transients for acto S1 dissociation and ADP affinity at 20 °C. A and C, the time course of fluorescence increase because of dissociation of S1 from pyrene-labeled actin by 10 (blue), 20 (green), and 40 μM ATP (red); the 30 μM trace (black) was omitted for clarity (A). Single exponential fits to the raw data gave the rates plotted at the four ATP concentrations in C. B and D, the increase of fluorescence with time for 0 ADP (red), 250 μM ADP (green), and 500 μM ADP (blue) in the presence of 200 μM ATP (B). The acto S1 was preincubated with the ADP before dissociation by the addition of ATP. The observed rates obtained from single exponential fits of these three and several other ADP concentrations are plotted in D. A hyperbolic fit of $k_{obs}$ to Equation 2 gave the dissociation constant, $K_{AD}$. These data are from experiment 1, wild-type α-S1.

The affinity of ADP for acto β-S1 ($K_{AD}$) could be determined for only one preparation of β-S1 because of the large amount of protein needed for this type of measurement (a separate sample of acto S1 has to be premixed with each concentration of ADP, which is then dissociated by a constant low concentration of ATP). Despite this limitation, it appears that the major difference between acto α-S1 and acto β-S1 is in the 4-fold greater affinity of ADP for the nucleotide-binding site of β-S1 compared with α-S1 (25 μM versus 100 μM, respectively) as evidenced by the $K_{AD}$ values in Table 2.

However, the rate of ADP release at a single saturating concentration of ADP (usually 200 μM ADP) with increasing concentrations of ATP could be determined for two independent preparations of wild-type β-S1 (Fig. 5 and Table 2), giving a $k_{-AD}$ value of ~130 s⁻¹. The difference between α- and β-S1 for the release of ADP in the presence of actin thus appears to be a ~3-fold slower rate with acto β-S1, consistent with the ADP affinity values.

Three independent preparations (including two different mouse lines) were used to compare R403Q α-S1 with its wild-type counterpart (Table 2). The remarkably consistent increase observed in $k_{-AD}$ for the mutant (430 s⁻¹) compared with the control (350 s⁻¹) gives us a high degree of confidence in the 20% change ascribed to the R403Q mutation ($p < 0.01$). In contrast, there was no statistically significant difference between the R403Q β-S1 and its wild-type control. However, it is noteworthy that paired experiments of mutant and control β-S1 measured within the same month did show a slight decrease in $k_{-AD}$ for R403Q β-S1 (Table 2). The low level of R403Q myosin expression in β isoform backbone precluded sufficient experiments for statistical analysis.

FIGURE 5. The ADP release rate from acto S1 at 20 °C. In the absence of added ADP nucleotide, the rates of acto S1 dissociation became too fast to measure at high ATP concentrations. They are approximately >1000 s⁻¹ for both α- and β-S1 isoforms (black and red symbols, respectively). With the addition of a constant concentration of ADP (usually 200 μM, but the same observed rates were obtained with 50 or 100 μM ADP), the rate constants for ADP release ($k_{-AD}$) for wild-type α-S1 and β-S1 differed by ~3-fold.

DISCUSSION
Here we present for the first time a transient kinetic analysis of mouse α- and β-cardiac myosin isoforms and the effect of a single point mutation on their biochemical properties. This study was made possible by the introduction of a His₆ tag along with the mutation in a transgenic construct containing cDNA for either α- or β-MHC (6). The R403Q mutation was chosen because it is one of the most lethal and well documented of the several hundred mutations reported for human cardiac myosin. We find that mouse cardiac myosins have faster transient kinetics than cardiac myosin from larger mammals (e.g., rabbits and pigs), and the data could be well fit by single exponentials.
(reviewed in Ref. 16). The ADP release rate and ADP affinity for the nucleotide-binding site differed by 3–4 fold between the slower β-S1 and the faster α-S1 wild-type isomorph.

Remarkably, a 4–5-fold difference in rates of cross-bridge detachment at high ATP (from which the ADP release rate can be obtained) was measured mechanically for α- and β-cardiac myosin isoforms in skinned mouse myocardial strips (17). The in vitro motility assay, a simplified model for actomyosin interactions, has consistently shown a 2-fold difference in velocities between the isolated cardiac myosin isoforms of individual species (18, 19). Considering the variety of approaches in experiments ranging from molecules to cells, the level of agreement for such a complex system is gratifying.

Importantly, we find that the R403Q mutation has little effect on the transient kinetic rates for mouse acto-β-S1 but showed a significant increase in the ADP release rate for mouse α-S1. These results corroborate our previous steady-state actin-activated ATPase activity measurements on the same isoforms isolated from transgenic mice (6).

Why Has It Been So Difficult to Resolve the Question of the Effect of a Single Point Mutation in Cardiac Myosin?—The only previous source of R403Q β-myosin has been from the cardiac and soleus (leg) muscles of patients with FHC (20). However, the small amount of protein isolated from biopsies limited functional assays to the in vitro motility assay, which showed an unusually large (~80%) decrease in actin filament velocity compared with controls. Expanding on this study with additional β-MHC gene mutations found in FHC patients (21), all seven mutant β-myosins showed reduced velocities, although most not as extreme as the R403Q mutation. Recognizing that these patients are heterozygotes (only one heavy chain in dimeric myosin is mutated), this strong inhibition suggested that some of the loss of movement may have been due to ATP-insensitive “rigor-like” actomyosin linkages. A subsequent motility study on R403Q myosin extracted from biopsies of the same patients sought to eliminate these noncycling rigor heads by extensive washing of the sample in the flow cell with unlabeled actin before applying the fluorescently labeled actin detected by the assay (22). Surprisingly, this modified approach now resulted in a small increase in actin filament movement. Perhaps the actin wash blocked many of the compromised mutant heads and thereby reduced the specimen to primarily native single-headed myosin with reduced strain dependence between heads. Whatever the explanation for the contradictory results, it is clear that more than one biophysical technique is needed to establish a definitive mechanism (reviewed in Ref. 2).

The Arg-403 residue is located at the base of a surface loop that lies at the actin-myosin interface. This site has been a “hot spot” for mutations, including a patient homozygous for the R403W mutation (23). Remarkably, this patient lived until middle age, when a heart transplant was needed. Myosin extracted from this tissue showed a slight increase in F-actin sliding velocity and actin-activated ATPase activity (23), but it should be emphasized that these results apply only to the tryptophan mutation and should not be extrapolated to glutamine. In the latter case, the R403Q mutation is lethal in homozygous embryos, and heterozygotes do not generally survive beyond a young age. This study illustrates dramatically what a major impact a single amino acid change at the same location can have on structure, function, and, ultimately, survival.

Ideally one would wish to screen a range of myosin mutations in an expression system. Unfortunately, this has proven difficult to accomplish: although smooth and nonmuscle myosins, and many unconventional myosins, have been expressed in good yield in the baculovirus insect cell system, it has not been possible to reproducibly express striated muscle myosins in this system. A promising approach was introduced by Winkelmann and co-workers (24, 25) with the development of a myogenic C2C12 cell line, which has been used recently for the expression of human skeletal and cardiac myosin subfragments (15, 26). The yields are still small from this expression system, and protein stability, particularly for mutants, remains to be established, but the early results are encouraging. Nonetheless, whether isolated from muscle fibers, expression systems, or transgenic animals, myosin remains an inherently complex, dynamic protein, and results should be interpreted with caution.

The Heavy Chain Backbone Defines the Functional Impact of the R403Q Mutation.—The His6 tag made it possible to isolate a heterodimeric α-MHC myosin, which contained one mutated R403Q α-head and one endogenous α-head. Although this myosin showed only a 20% increase in velocity in the motility assay, this result was fully consistent with the >40% increase found for a homodimeric R403Q/R403Q myosin isolated from a homozygous mouse generated by homologous recombination (4). Besides confirming that a gain of function in mouse α-cardiac myosin activity can be produced by the lethal R403Q mutation, it was important to demonstrate this effect in myosin isolated from an adult mouse that lived to maturity, instead of surviving only 1 week after birth, as do homozygous mice (4).

However, the major goal in introducing a mutation by transgenesis was the ability to isolate a mutant cardiac myosin in a β-MHC backbone. Unfortunately, the heterodimeric myosin in this instance has a R403Q β-head coupled to an endogenous α-MHC head, thus confounding the interpretation of in vitro motility data. However, by proteolytic digestion of myosin, it was possible to isolate a homogeneous population of S1 heads for comparison by steady-state actin-activated ATPase activity. We found that the mutation in α-S1 increased the activity by ~30%, consistent with the in vitro motility assay, but the unanticipated new finding was the lack of any enhanced steady-state enzymatic activity for the R403Q β-S1, in fact, a slight loss of ATPase activity was observed (6). Because a disease mutation can be expected to cause only minor perturbations in protein structure and function, another assay system was essential to reach any firm conclusions.

Here we show by stopped flow kinetics that the mutation in β-S1 does indeed have a phenotype different from that observed in α-S1: whereas R403Q caused no significant change in the release of ADP from β-S1, a ~20% increase in k_−AD was found for α-S1 (p < 0.01). These findings corroborate our earlier conclusion from steady-state ATPase kinetics that the heavy chain backbone of myosin has a profound effect on function. The use of transient kinetics has important advantages over a steady-state assay: the stopped flow assay is relatively insensitive to minor contaminants or denatured S1 heads, and a

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single major amplitude implies a homogeneous population of molecules. In contrast, ATPase activity measures the entire population of myosin molecules, and the final value is dependent on protein concentration. By measuring chromatographically purified α-S1 and β-S1 in parallel by stopped flow kinetics, experimental error is minimized, and a high level of confidence in the results can be achieved.

Conclusions—We find in agreement with earlier studies that the R403Q mutation in a mouse α-MHC backbone results in a gain of function, a model that has been widely accepted as the primary cause leading to hypertrophic cardiomyopathy. However, we find that this enhancement does not extend to the R403Q mutation in a mouse β-MHC backbone. Our results do not necessarily imply a universal mechanism for FHC mutations in the β-myosin heavy chain. The location of a mutation is undoubtedly important, and the functional effect could be species-dependent. It has been shown that the maximum shortening velocity of single slow skeletal muscle fibers (containing essentially the β-myosin isoform) is 5-fold faster in the mouse than in the corresponding human fibers (27). The reason for this large difference in velocity is not readily apparent because the orthologous β-myosins from these two species are >98% identical in sequence, and there are no differences in the surface loops usually associated with function (28).

In an effort to minimize species-dependent differences further, a transgenic rabbit has been generated that expresses predominantly β-myosin in its ventricles. We find that the R403Q mutation in this rabbit cardiac myosin results in a significant reduction in actin filament movement by the in vitro motility assay, and a slight reduction in actin-activated ATPase activity.3 Overall, these findings led us to predict that the R403Q mutation will probably lead to a decline in functional properties in the hearts of all larger mammals, including humans, even though the rates of transition between states in the kinetic cycle are expected to vary across species (29).

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