Cardiac Myosin Isoforms from Different Species Have Unique Enzymatic and Mechanical Properties†

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Received March 9, 2004; Revised Manuscript Received September 14, 2004

ABSTRACT: The mammalian heart contains two cardiac myosin isoforms: β-myosin heavy chain (MHC) is found predominantly in the ventricles of large mammals, and α-MHC is expressed in the atria. The sequence identity between these isoforms is ∼93%, with nonidentical residues clustered in discrete, functionally important domains associated with actin binding and ATPase activity. It is well-established that rabbit α-cardiac myosin has a 2-fold greater unloaded shortening velocity than β-cardiac myosin but a 2-fold lower average isometric force. Here, we test the generality of these relationships for another large mammal, the pig, as well as for a small rodent, the mouse, which expresses α-MHC in its ventricles throughout adulthood. Hydrophobic interaction chromatography (HIC) was used to purify myosin from mouse, rabbit, and pig hearts. The superior resolving power of HIC made it possible to prepare highly homogeneous, enzymatically active myosin from small amounts of tissue. The movement of actin filaments by myosin was measured in an *in vitro* motility assay. The same assay could be used to determine average isometric force by loading the actin filaments with increasing concentrations of α-actinin to stop filament motion. We conclude that myosin from the mouse has significantly higher velocities for both α and β isoforms than myosin from rabbits and pigs, even though the 2-fold difference in velocity between isoforms is maintained. Unlike the larger mammals, however, the small rodent generates the same high isometric force for both α and β isoforms. Thus, nature has adapted the function of cardiac myosin isoforms to optimize power output for hearts of a given species.

Heightened interest in cardiac myosin has been generated by the finding that many cardiac diseases, such as familial hypertrophic cardiomyopathy (FHC)[1] and dilated cardiomyopathy (DCM), are caused by point mutations in the β-cardiac myosin heavy chain (MHC) (over 30% of FHC cases are accounted for by this gene, reviewed in ref *1*). The mechanism by which these mutations cause disease is thought to involve fundamental alterations in the way cross bridges produce force and movement in a muscle. Despite the large number of biochemical and biophysical studies related to FHC mutants over the past decade, no consensus has been reached on the functional consequences of mutations in β-MHC. In some instances, studies on myosin containing identical point mutations have led to opposite conclusions (reviewed in ref *2*). Some of these discrepancies can probably be ascribed to the relative instability of cardiac myosin in dilute solutions, particularly when prepared from small amounts of heart tissue from transgenic mice, a common source of mutant proteins, or from muscle biopsies of patients with cardiac failure (*2*).

Myosin exists as two isoforms in the myocardium: in large mammals, β-MHC is found primarily in the ventricles (but is also expressed in slow skeletal muscle fibers), and α-MHC is found in the atria. The amino acid sequence identity between α- and β-MHC is ∼93% (*3*), with nonidentical residues clustered in discrete, functionally important domains (*4*). The isoform composition varies during development and can be shifted by various stimuli such as thyroid hormone and pressure overload (*5*). It is well-established that the unloaded velocity of shortening for an α-MHC isoform is 2−3-fold greater than for a β-MHC isoform (*3*) of the same species, whether measured in myocardial preparations (*6*) or by an *in vitro* motility assay in which actin filaments glide over monomeric myosin adhered to a substratum (*7*). However, absolute rates of ATPase activity or actin filament velocity vary widely among species (see Table 1), with small mammals exhibiting significantly higher rates.

In an effort to determine more precisely how motor function varies with species, we have compared the enzymatic and mechanical properties of α- and β-myosin isoforms isolated from the hearts of mice, rabbits, and pigs, animals that have been shown to vary in their isoenzymic patterns during development. Mice express predominantly α-myosin in the ventricle throughout adult life, whereas rabbits and

† This work was supported by NIH Grants AR47906 and HL59408 to S.L. and the Swedish Medical Research Council, the Swedish Society of Medicine, and Goijes Minne to U.P.M.

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[1] Abbreviations: HIC, hydrophobic interaction chromatography; MHC, myosin heavy chain; S1, myosin subfragment 1; FHC, familial hypertrophic cardiomyopathy; DCM, dilated cardiomyopathy; PTU, propylthiouracil; FPLC, fast performance liquid chromatography; NEM, *N*-ethylmaleimide; pPDM, *N,N′-p*-phenylenedimaleimide.

10.1021/bi0495329 CCC: $27.50 © 2004 American Chemical Society
Published on Web 11/04/2004

Table 1: Comparison of Enzymatic Rates and Actin Filament Velocities for Cardiac Myosin Isoforms from Several Species[a]

myosin heavy chain	actin-activated ATPase (s^{-1})	actin velocity (μm/s)	reference
human β	0.32 (30 °C)	1.2	*40*
	nd	1.4	*19*
	nd	0.48 (25°C)	*41*
	nd	1.5	*9*
human α	nd	2.0	*9*
rabbit β	1.6 (30 °C)	1.1	*24*
	3.1 (30 °C)	1.6	*7*
	nd	1.8[b]	*9*
	0.67 ± 0.04 (27 °C)	2.0 ± 0.2	this study
rabbit α	5.6 (30 °C)	2.9	*24*
	5.7 (30 °C)	4.6	*7*
	nd	2.8[b]	*9*
	1.41 ± 0.03 (27 °C)	4.0 ± 0.4	this study
pig β	nd	1.8 ± 0.2	this study
pig α	nd	3.8 ± 0.5	this study
mouse β	nd	2.6	*3*
	nd	2.6 ± 0.4	this study
mouse α	1.6 (RT)	5.5	*42*
	nd	5.5	*3*
	1.8 ± 0.04 (27 °C)	5.1 ± 0.9	this study

[a] Temperatures for ATPase rates are given in parentheses. Actin filament velocities are at 30 °C unless noted otherwise. RT, room temperature; nd, not determined. [b] Regulated thin filaments. Values represent the mean ± SD. This table is limited to enzymatic rates determined since the 1990s, when motility values became more widely measured.

pigs have primarily β-myosin in the adult (*8*). However, even a small rodent such as the rat shows a significant increase in β-MHC expression with aging (*6*). To reduce the variability inherent in myosin preparations, purification methods were developed to maximize stability and homogeneity. We find that even though a 2-fold difference in actin filament velocity (V_{actin}) is maintained between α- and β-cardiac myosin of all mammalian species, the difference in average isometric force (F_{avg}) between myosin isoforms depends markedly upon the species. In the mouse, the α- and β-cardiac isoforms have the same isometric force, which is equivalent to that found for β-cardiac myosin in all mammals. In contrast, rabbit α-cardiac myosin exerts only half the force of rabbit β-cardiac myosin. A lower force for α-cardiac myosin was also found in the pig and is probably representative of all larger mammals including humans (*9*). These results emphasize how small differences in sequence among highly homologous myosins (>95% sequence identity between α-MHC of the mouse and pig) can have a disproportunately large effect on function (*3*, *10*). Thus, caution should be exercised in extrapolating observations made from transgenic mice expressing α-cardiac myosin with FHC or DCM mutations to humans with cardiomyopathies triggered by mutations in their β-cardiac myosin. The effect of a mutation in a different myosin backbone may not necessarily have the same physiological consequences in all species.

EXPERIMENTAL PROCEDURES

Experimental Animals. Adult mice express predominantly α-MHC in their ventricles; therefore, the animals were treated with propylthiouracil (PTU) to shift expression to the β-MHC isoform (*3*). Conversely, rabbits were treated with L-thyroxine to replace their native β-MHC with a homogeneous population of α-MHC (*7*). After the animals were sacrificed, the isolated ventricles were frozen in liquid nitrogen and stored at −80 °C. Adult rabbit ventricles, mouse hearts, and pig hearts were also obtained from Pel-Freez Biologicals. Adult pigs have primarily β-MHC in their ventricles, and the atrium was used as a source of α-MHC. Chicken gizzards from Pel-Freez were used for the preparation of smooth muscle myosin (*11*).

Protein Purification. When myosin was prepared from a relatively large amount of tissue (e.g., three or more rabbit hearts), the purification procedure followed essentially that described for skeletal muscle myosin (*12*) with minor modifications. The cardiac tissue was homogenized in 40 mM imidazole at pH 7.2 and 2 mM $MgCl_2$ (Sorvall Omnimixer), and the muscle mince was washed in the same buffer until the supernatant was colorless. The pellet was homogenized in the extraction buffer [0.3 M NaCl, 150 mM sodium phosphate at pH 6.5, 5 mM $MgCl_2$, 2 mM ATP, 2 mM dithiothreitol (DTT), 1 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and protease inhibitors (5 μg/mL leupeptin, 0.5 mM AEBSF, and 0.5 mM TLCK], and the suspension was stirred for about 15 min. After centrifugation, the supernatant was diluted 15−20-fold with water containing 1 mM DTT. The precipitated protein was collected by centrifugation, and the pellet was dissolved in 0.6 M KCl, 25 mM sodium phosphate at pH 7.0, 2 mM $MgCl_2$, 1 mM DTT, and 1 mM NaN_3 against which it was dialyzed overnight in the cold. To minimize contamination by actin, 2 mM MgATP was added to the preparation, which was centrifuged for 3 h at 45 000 rpm. The supernatant was dialyzed against 20 mM sodium pyrophosphate at pH 7.5 and 0.5 mM DTT overnight with two changes and chromatographed by DEAE-Sephacel ion-exchange chromatography as described in Margossian and Lowey (*12*). After concentration, the myosin was stored at −20 °C in buffered high salt containing 50% glycerol.

Before any biochemical or mechanical assays, ∼10 mg of the stored myosin was further purified by hydrophobic interaction chromatography (HIC) on a 1 × 30 cm column of Toyopearl ether-650S resin (Tosoh Bioscience) equilibrated in 1.4 M ammonium sulfate, 20 mM imidazole at pH 6.8, 2 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM DTT, 0.5 mM ATP, and 3 mM NaN_3 (buffer A). The myosin was eluted with a 100 mL gradient of ammonium sulfate going from 1.4 to 1.2 M. To program the gradient on the AKTA fast performance liquid chromatography (FPLC) system (Amersham Biosciences), buffer B had the same composition as buffer A minus the ammonium sulfate. Fractions were analyzed by SDS−PAGE, and pools were concentrated by dialysis against 4−5 volumes of saturated ammonium sulfate (*13*). Nondenaturing gel electrophoresis was used to establish the myosin isoform composition (*11*). The concentration of myosin was determined using an extinction coefficient of 0.5 cm^{-1} for 1 mg/mL at 280 nm or by the colorimetric method of Bradford using myosin as a standard.

For smaller amounts of tissue (∼10 mouse hearts or <1 g), the procedure was modified so that the washed muscle mince was extracted in 0.3 M NaCl, 10 mM sodium pyrophosphate, 150 mM sodium phosphate at pH 6.8, 1 mM DTT, 2 mM $MgCl_2$, 1 mM EGTA, and protease inhibitors. The protein was precipitated and collected as above, but

instead of ion-exchange chromatography, the protein was applied directly to a 1 × 6.8 cm (~5 mL) prepacked Toyopearl MD-G Ether column for HIC chromatography. Using this procedure, it was possible to isolate ~1−2 mg of highly purified cardiac myosin.

Actin-Activated ATPase. The actin-activated MgATPase activities of all myosin species were determined at 27 °C in 50 mM KCl, 1 mM $MgCl_2$, 2 mM MgATP, 10 mM imidazole at pH 7.5, 1 mM DTT, and 1 mM NaN_3. A few measurements were performed at 37 °C. Actin concentrations ranged from 2.5 to 40 μM (details in ref *11*). The data were fitted to Michaelis−Menten kinetics.

Analytical Ultracentrifugation. A Beckman Optima XL-I analytical ultracentrifuge was used to determine the sedimentation velocity profile of the myosin isoforms. The runs were performed in the An60Ti rotor at 50 000 rpm and 20 °C. Samples of ~0.5 OD in a 1.2 cm cell were scanned by absorbance at 280 nm. Sedimentation boundaries were analyzed by time-derivative analysis software (*14*).

In Vitro Motility Assays. The unloaded velocity of actin filaments and the relative isometric force of myosin isoforms were determined at 30 °C using the *in vitro* motility assay. The procedure for the unloaded velocity measurements was essentially as described previously (*15*, *11*). The myosin samples at a final concentration between 0.02 and 0.16 mg/mL were centrifuged with an eqiumolar concentration of F actin in the presence of 1 mM MgATP to remove any remaining ATP-insensitive rigor heads before infusion into the flow cell. In some experiments, this step was omitted and the HIC chromatographed myosin was applied directly to the flow cell. The standard assay buffer (A) contained 25 mM KCl, 25 mM imidazole at pH 7.5, 1 mM EGTA, 4 mM $MgCl_2$, and 10 mM DTT. The final assay buffer contained in addition 0.5% methylcellulose, oxygen scavengers, and 1 mM MgATP (*11*). The average isometric force was determined by placing an internal load on the actin filaments using α-actinin (*16*). As α-actinin (Sigma) binds to actin, filament velocity is slowed; when the load on actin is comparable to the force-generating capacity of myosin, filament movement will stop. Isometric force is defined as the minimum concentration of α-actinin needed to completely stop filament motion at any given myosin concentration. The greater the force on the actin filament the more α-actinin is needed to stop movement. Thus, the concentration of α-actinin needed to stop movement is dependent on the myosin concentration and the force-generating capacity of the myosin. For determination of the relative force, the proteins were infused into the flow cell as follows: myosin was infused in buffer A with added salt, and after 1 min, the chamber was washed 3 times with buffer A; then, α-actinin was infused, and after 1 min, the flow cell was washed 3 times with buffer A containing the blocking agent bovine serum albumin. Fluorescently labeled actin was infused for 30 s with one repeat, then the chamber was washed twice with a rigor solution, and finally motility was initiated by addition of the rigor solution containing 1 mM MgATP (*11*). The movement of actin filaments was observed using a Zeiss IM microscope with fluorescence illumination and recorded on videotape. Filament velocities were calculated as previously described (*17*). The velocities of 20−30 filaments were averaged to obtain the mean ± standard deviation (SD).

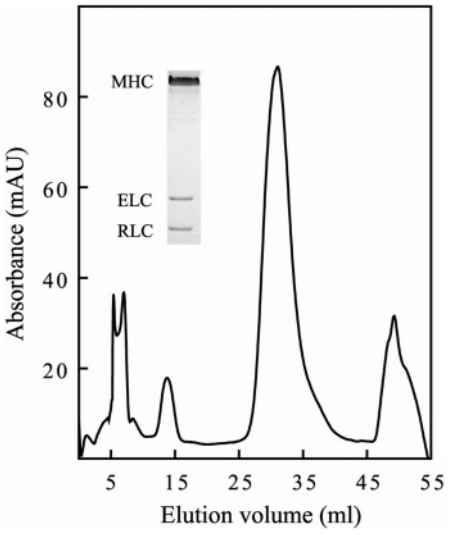

FIGURE 1: Isolation of cardiac myosin by HIC. Applying ~5 mg of crude β-cardiac myosin to a 1 × 6.8 cm prepacked Toyopearl MD-G Ether column, we recovered ~1−2 mg of pure myosin by elution with a gradient of decreasing ammonium sulfate concentration from 1.4 to 1.2 M. The inset shows an SDS−PAGE gel of the major pooled fractions (about 6 mL).

RESULTS

Enzymatic Activity and Motility. Our studies focused initially on cardiac myosin from mature rabbit hearts, because the isoform in adult ventricles is predominantly β-MHC, with little α-MHC present. Rabbits that were treated with PTU to ensure a homogeneous population of β-MHC isoform gave essentially the same results. When using several frozen rabbit hearts, the protein was initially prepared by the procedures used for skeletal myosin (*12*). However, myosin isolated from frozen tissue is generally contaminated by more actomyosin and impurities than myosin prepared from fresh tissue, and therefore, additional chromatography is needed to ensure a high level of purity (see the Experimental Procedures). We have found that HIC, which is based on the hydrophobicity profile of a protein, is far superior to ion-exhange chromatography in resolving power (*18*). Particularly in dealing with small amounts of protein (extracted from one rabbit heart or several mouse hearts), HIC columns have shown excellent separation of myosin from contaminating proteins (Figure 1). When analyzed by sedimentation velocity in the analytical ultracentrifuge, the HIC-purified native myosin showed a single symmetrical peak with no aggregates, evidence for a homogeneous macromolecular solution (Figure 2).

Prior to the standard motility assay, myosin is usually centrifuged with an equimolar concentration of F actin in the presence of millimolar MgATP to remove any "rigor-like" myosin that cannot dissociate from actin. Such denatured myosin acts as a load in the motility assay to retard the movement of fluorescently labeled actin filaments. Some investigators, in addition, introduce short unlabeled actin filaments directly into the flow cell to ensure the complete absence of any of these rigor-like molecules (*19*). A remarkable feature of HIC-purified myosin is that smooth movement of all of the tetramethyrhodamine-labeled actin filaments is observed even without any spin down with actin. *In vitro* motility data for HIC-purified preparations of rabbit, mouse, and pig α- and β-MHC isoforms are shown in Figure 3. In all cases, myosin purified only by precipitation at low

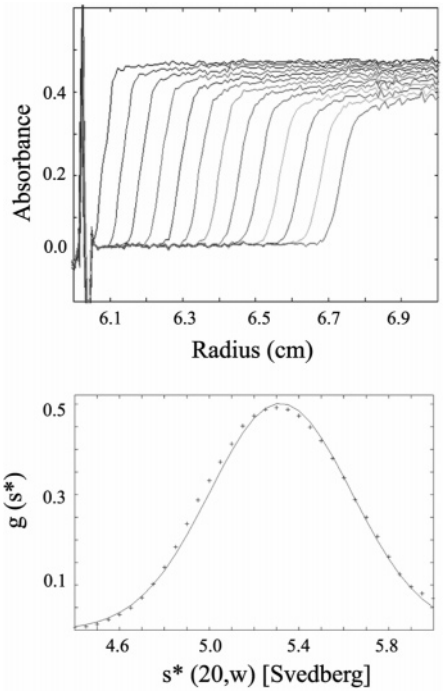

FIGURE 2: Characterization of rabbit cardiac myosin by sedimentation velocity. The top panel shows the absorbance of the protein as a function of the distance and time in the ultracentrifuge cell. The lower panel shows the distribution, $g(s^*)$, of sedimentation coeficients as analyzed from the sedimentation velocity data above. The good fit of the data to a single species demonstrates the homogeneity of the protein preparation (*14*).

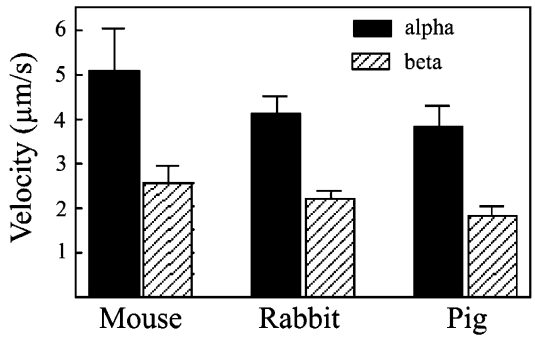

FIGURE 3: Actin filament velocities for mouse, rabbit, and pig cardiac myosin isoforms. Values are mean velocities (μm/s) ± SD for at least two independent preparations of mouse α-MHC (5.1 ± 0.9) and β-MHC (2.6 ± 0.4), rabbit α-MHC (4.1 ± 0.4) and β-MHC (2.2 ± 0.2), and pig α-MHC (3.8 ± 0.5) and β-MHC (1.8 ± 0.2). For each value, 40−50 filaments were measured. Earlier measurements for the rabbit isoforms gave similar values.

ionic strength or by ion-exchange chromatography gave lower values for motility than HIC-purified preparations.

These same HIC-purified myosins were measured for their actin-activated MgATPase activity. Data from four to five independent preparations of rabbit α- and β-cardiac myosin obtained at 27 °C are shown in Figure 4. The scatter in the data is largely a consequence of mixing two filamentous protein systems for the assay. However, the average value for V_{max} from these data and several other preparations remained consistently at 1.4 and 0.7 s^{-1} for α- and β-rabbit cardiac myosin, respectively. Similar determinations for mouse α-cardiac myosin and chicken pectoralis myosin gave values of 1.8 and 4.7 s^{-1}, respectively. Because the K_m for myosin was <5 μM and the range of actin concentrations used in the measurements extended to 40 μM, a reliable

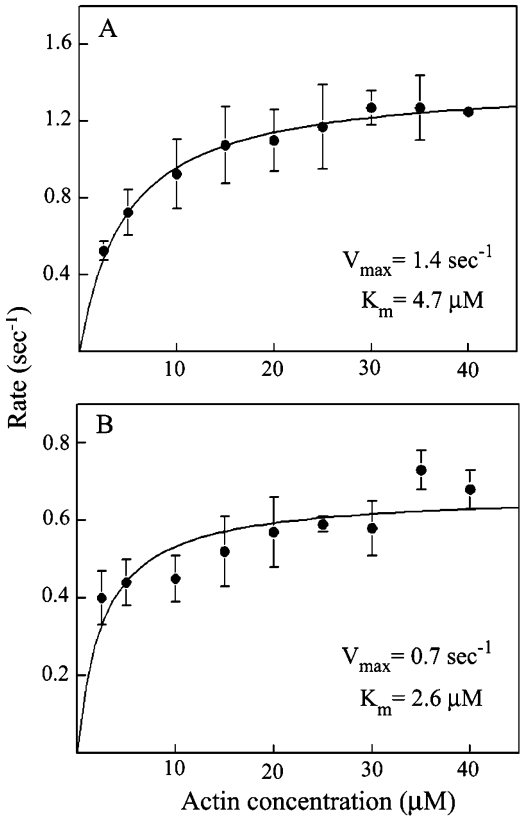

FIGURE 4: Actin-activated MgATPase activity for rabbit α- and β-cardiac myosin. The rate of ATP hydrolysis was measured for four independent preparations of rabbit α-MHC (A) and five preparations of rabbit β-MHC (B) isoforms. The mean ± SD is shown at each actin concentration. The rate in the absence of actin was subtracted from the values, and therefore, the fit must go through the origin. The line is the best fit of the data to the Michaelis−Menten equation. Some scatter in the data is unavoidable when measuring rates for two filamentous protein systems. However, an advantage of using native myosin rather than S1 is the low K_m, which makes extrapolation to V_{max} more precise.

extrapolation to V_{max} was possible by fitting the data to the Michaelis−Menten equation. A limited number of measurements at 37 °C gave a 2−3-fold higher rate, consistent with other reports (*20*). Actin-activated ATPase values reported for rat cardiac myosin isoforms fell well within the range reported here (*21*).

A stringent test for the relative accuracy of these measurements is to plot the actin-activated MgATPase activity (V_{max}) against actin filament velocity (V_{actin}). If these proteins obey the basic tenet of muscle physiology first proposed by Barany in 1967 (*22*), namely, that actomyosin activity is proportional to the velocity of unloaded muscle shortening, the mechanical and enzymatic properties should be coupled and a linear relationship should be obtained. Figure 5 shows this to be the case: the parameters characterizing α- and β-rabbit cardiac MHC, α-mouse cardiac MHC and chicken pectoralis myosin, measured under identical conditions, fall on a single line within experimental error. Phosphorylated smooth muscle myosin was taken from an earlier study (*23*), but the low rates for this myosin have little influence on the slope, V_{actin}/V_{max}.

Average Force Determinations. Two methods are currently in use for determining the average isometric force from a conventional *in vitro* motility assay: in the "mixture protocol", one myosin is mixed in different proportions with

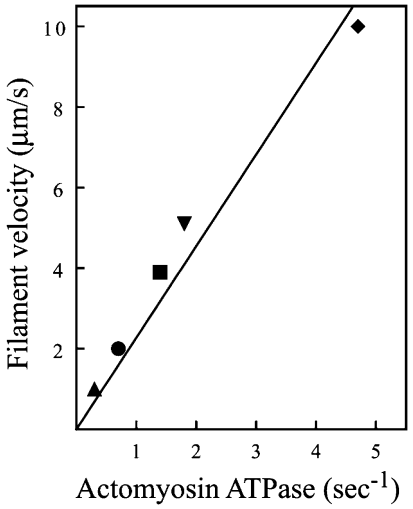

FIGURE 5: Coupled linear relationship between V_{actin} and the steady-state actin-activated MgATPase activity for several muscle myosin isoforms. The values for rabbit α-MHC (■) and β-MHC (●), mouse α-MHC (▼), and chicken skeletal myosin (◆) were determined under identical conditions. The smooth muscle myosin data (▲) are from an earlier study (*23*). No error bars are included, because the number of determinations varied among the different isoforms, but a SD of ∼15% is reasonable.

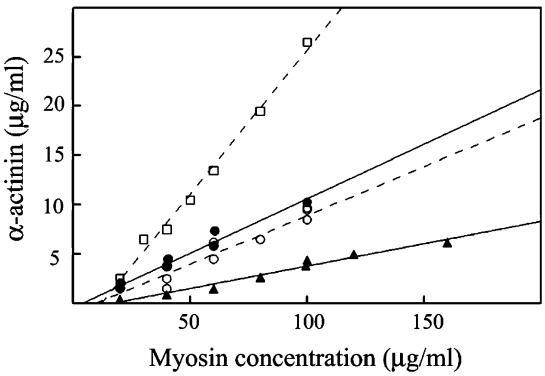

FIGURE 6: Average isometric force generated by different myosin isoforms in an *in vitro* motility assay. The actin-binding protein, α-actinin, was added to the standard motility assay to act as an internal load on the movement of the actin filaments. At each myosin concentration, the "critical concentration" of α-actinin needed to stop the actin filaments from moving was determined. The values for the relative isometric force can be determined from the slopes; if we normalize the slope of rabbit β-cardiac myosin (●) to 1, then chicken skeletal myosin (○) is 0.8, rabbit α-cardiac myosin (▲) is 0.4, and chicken smooth muscle myosin (□) is 2.6. The values for F_{av} from ≈3 to 1 to 0.5 for smooth > skeletal ≈ β-cardiac > α-cardiac agree well with earlier measurements using the ultracompliant glass needle assay.

a second, slower myosin, and the motility of the mixture is determined. From the shape of the plot, the relative average force (F_{avg}) can be estimated. This type of analysis is model-dependent and based on the mechanical interactions between two proteins (*24*). However, the relative isometric force determined by this approach has been in remarkably good agreement with the more direct but laborious bending needle assay (*7*, *25*). An alternative method has been to use the α-actinin interaction with actin as a internal load against which the myosin has to work to produce movement (*9*, *16*). At each myosin concentration, the "critical concentration" of α-actinin needed to stop the actin filaments from moving is determined (Figure 6). The linearity of this plot is a strong indication that this assay is actually measuring a restraining force. When the slopes for different isoforms were compared, the relative average force for β-rabbit cardiac myosin was ∼2-fold greater than for α-rabbit cardiac myosin and the average force for smooth muscle myosin was ∼3-fold greater than that of skeletal or β-cardiac myosin. These relative values are in remarkably good agreement with earlier methods that have been used for measuring relative isometric forces for smooth, skeletal, and cardiac myosins isoforms (see the Discussion). To further test the reliability of this method as an indicator of relative isometric force, we used several analogues of ATP, which are known to reduce the force levels and unloaded shortening velocities of skinned rabbit fibers (*26*). We were gratified to see a qualitatively similar drop in force and actin filament velocity for α-mouse cardiac myosin in the presence of UTP and ITP compared to ATP (The actin filament velocity in UTP and ITP dropped to 1.8 and 1.2 μm/s, respectively, compared to 4.7 μm/s in ATP. The relative isometric force compared to ATP was 0.8 for UTP and 0.6 for ITP).

We were interested in determining the F_{avg} of another species to see if the 2-fold relationship between α- and β-rabbit cardiac myosins was a general phenomenon for all species. Unexpectedly, α-mouse cardiac myosin had the same force as β-mouse cardiac myosin (Figure 7). Moreover, the values for the mouse isoforms fell on the same plot as the values determined for rabbit β-myosin and chicken skeletal myosin, suggesting they all share the same average isometric force. Because rabbit α-cardiac myosin was the only cardiac isoform with a distinctly lower F_{avg}, we examined the myosin isoforms from another large mammal. The pig, like the adult rabbit, expresses primarily β-cardiac myosin in its ventricles. The atria were used as a source of α-myosin (*27*). These isoforms showed the same 2-fold relationship in F_{avg} as the rabbit (Figure 7), suggesting that the mouse isoforms were the exception. It is noteworthy that the mouse isoforms also have distinctly higher actin filament velocities than the myosin isoforms from the larger mammals, even though the 2-fold relationship between the α- and β-cardiac myosins is maintained. An important feature of these measurements is that the various myosin preparations were all chromatographed on HIC columns, and the assays were performed on fresh material by identical procedures, thereby minimizing any errors in a comparison.

DISCUSSION

Mutations in myosin can lead to a wide spectrum of heart disorders that vary in severity. Some mutations cause very mild symptoms or go undetected (particularly because patients are heterozygous for the mutation). Thus, the functional consequences of many of the more than 50 point mutations in myosin are expected to be minor at the molecular level and difficult to detect by *in vitro* assays. This makes it especially more important to have a uniformly pure and active preparation of cardiac myosin for biochemical characterization, to avoid having small changes masked by variations in the preparation. Much of what has been learned about muscle myosin or "conventional myosin" over the past 40 years has been from protein isolated from rabbit psoas or chicken pectoralis muscles. These preparations and their proteolytic subfragments are routinely purified by ion-exchange chromatography and, more recently, in the case

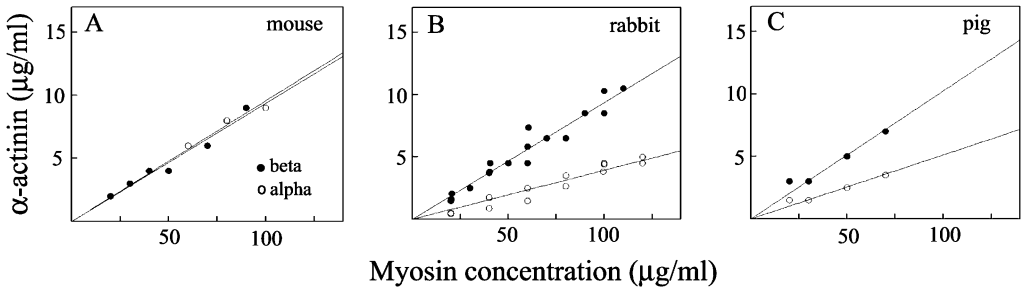

FIGURE 7: Average isometric force generated by the α and β isoform of three different species. (A) Mouse α and β isoforms have the same F_{av}. (B) Slopes for the rabbit isoforms are 1−0.4 as in Figure 6. (C) Slopes for the pig isoforms are 1−0.5.

of expressed fragments, cloned with a tag, by affinity chromatography. Cardiac myosin has the reputation of being less stable and more susceptible to proteolytic degradation than skeletal myosin, and most "purification" procedures have been limited to extraction at high ionic strength followed by dilution with water to precipitate the myosin. Here, we have purified cardiac myosin by hydrophobic interaction chromatography and compared the enzymatic and mechanical properties of α- and β-MHC isoforms from several species. Literature values representative of the activity of α- and β-cardiac myosins from earlier studies are summarized in Table 1, which also includes the values determined in the present study.

Enzymatic Cycle and the Duty Ratio. It is apparent that there is a far greater disparity in ATPase rates of cardiac myosin than in actin filament velocity. Besides variations in experimental conditions (temperature, pH, and salt concentration), which will all impact the rate, there is most likely some heterogeneity in the myosin preparations, judging from the chromatogram in Figure 1. Any error in the myosin concentration will be reflected in the maximum rate. Impurities have less influence on the velocity determinations, because they are relatively independent of the myosin concentration but are primarily affected by rigor-like molecules, which retard actin filament movement. Because methods have been developed that minimize the effect of these rigor-like molecles, it is possible to conclude from the data in Table 1 that β-cardiac myosin from large mammals moves actin filaments with a velocity between 1.5 and 2.0 μm/s at 30 °C. The α-cardiac myosin from these same species moves actin with a 2-fold higher velocity of 3−4 μm/s. The exception to these values is encountered in the mouse, which has a significantly higher velocity of movement for β- and α-cardiac myosin, 2.6 and 5.5 μm/s, respectively.

The biochemical ATPase rate and the mechanical filament velocity can be linked by a concept known as the duty ratio (*28*). If we define the duty ratio (f) as the fraction of the cross-bridge cycle time that myosin is strongly attached to actin following its power stroke, then $f = t_{on}/t_c$, where t_{on} is the time of the attached state and t_c is the total cycle time of ATP hydrolysis or $1/V_{max}$. If we further assume $V_{actin} \sim d_{uni}/t_{on}$, where d_{uni} is the unitary displacement or step size generated by the power stroke, then $f = d_{uni}V_{max}/V_{actin}$. When 1/slope in Figure 5 is multiplied by the step size, taken as ∼10 nm for class II myosins (*29*), an f of <1% is obtained. This value is within the range of unloaded duty ratios reported in the literature (*24*, *29*), although lower than the value (∼5%) derived from motility data alone (*30*, *31*). The duty ratio from motility assays depends on a number of assumptions including (1) that cross-bridge interactions with actin are independent of myosin orientation in the flow cell, and (2) that the number of interacting myosin molecules on the coverslip can be accurately determined by comparing the myosin ATPase activity on the coverslip to a standard activity curve in solution. The duty ratio is inversely related to the minimum number of myosin heads (N) required for maximum movement, $N \approx 1/f$, or ≈20 for an $f = 5\%$. This number seems small considering the large size of a myosin filament in a muscle cell. The duty ratio obtained from coupling the biochemical and mechanical cycles in Figure 5 leads to a considerably larger number of myosin heads ($N \approx 200$), but this method is not without its limitations. ATPase activity is difficult to measure for filamentous systems, primarily because of the mixing problems in a viscous solution. Because all of the myosin heads may not interact with actin, the data will probably lead to an underestimation of the duty ratio. One could perform the activity measurements with soluble proteolytic subfragments (which are commonly used for transient kinetic analyses), but proteolytic digestion may affect the activity. Although HMM (the double-headed fragment of myosin) has a somewhat higher actin-activated ATPase activity than native myosin, S1 (the single-headed subfragment) can have as much as a 5-fold higher ATPase activity (*32*, *33*). This is especially evident for S1 prepared by digesting myosin with chymotrypsin, which results in the loss of the regulatory light chain. But more importantly, subfragments do not lend themselves to motility assays because of unfavorable interactions with the substratum. We have considered native myosin as the best compromise for both types of measurements but recognize that the true duty ratio probably lies between the extremes of 1 and 5%, with the lower range favored by our data.

Average Force Comparison. The concept of the duty ratio (or duty cycle) was first introduced to explain the higher isometric force generated by smooth muscles despite their lower content of myosin relative to skeletal muscles (*34*). A high duty ratio for smooth muscle myosin under loaded, isometric conditions (f_{iso}) would yield a greater average force than for skeletal muscle myosin ($F_{avg} = f_{iso}F_{uni}$), where F_{uni} is the unitary force generated by myosin. The most direct measurement of average force was made by observing the bending of a calibrated, ultracompliant glass microneedle as it attaches to an actin filament that is moving across a myosin-coated surface (*35*). Using this method, the average force of smooth muscle myosin was shown to be approximately 3 times that of skeletal muscle myosin (*25*). Similarly, the average isometric force of β-rabbit cardiac

myosin was shown to be approximately twice that of α-rabbit cardiac myosin (*7*). A less direct but simpler motility assay for determining the relative average force is to mix two myosins with different cycling rates and observe the actin filament velocities as the slower myosin imposes a load against which the faster myosin must move (*15*, *24*). Despite its model dependence and a number of assumptions (see ref *24* for a detailed discussion), the ranking of average myosin forces by this assay, smooth > skeletal ≈ β-cardiac > α-cardiac (*24*), agrees remarkably well with results obtained from the more direct microneedle assay (*7*, *25*).

We have used a modified version of the "mixture protocol" described above to compare relative isometric forces by imposing an internal load on the actin filament to stop all filament movement by myosin. This approach was first introduced by Warshaw et al. (*15*) who showed that noncycling NEM- or pPDM-treated myosin could inhibit filament movement by attaching to actin. Instead of using chemically modified myosin, whose properties tend to change with time, we have found that α-actinin, a highly α-helical actin-binding protein, whose properties remain constant for over a year, is very effective at stopping actin filament movement (*16*). The amount of α-actinin needed to inhibit motion is linearly related to the concentration of myosin heads in the flow cell and to the average force exerted by these myosin molecules. When the experimental slopes for smooth muscle myosin, α- and β-rabbit cardiac myosin and chicken skeletal myosin were compared, we found that the relative average isometric forces were the same as those obtained by the microneedle assay. Encouraged by the good agreement between these different methods, we used the α-actinin approach to compare the isometric force levels of α- and β-mouse cardiac myosin. Given the extensive use of transgenic mice as a model system in studies of FHC, it is important to have reliable values for the mechanical properties of native mouse cardiac isoforms. To increase the level of confidence in our results, we simultaneously measured the relative force of myosins isolated from the atria and ventricles of the pig. We find that the pig isoforms, like the rabbit isoforms, differ 2-fold in force, but the mouse isoforms are more similar to the rat isoforms (*36*, *37*) in that their α- and β-cardiac myosins have identical forces. It appears that the 2-fold lower force of rabbit α-cardiac myosin is a feature common to all larger mammals but not shared by small rodents (*3*). Even though the primary structure, ∼95% sequence identity between mouse and rabbit α-cardiac myosin, of these myosins is so highly conserved, they generate quite different force levels. A challenge for the future will be to understand what structural features of the myosin molecule are responsible for generating these large differences in mechanical and enzymatic properties.

Physiological Significance. Physiological measurements of the force-generating properties of myofibrils from mouse, guinea pig, and human ventricles showed similar force levels for these species, implying that α-MHC in the mouse has the same force as β-MHC in humans (*38*). Studies using rat myocardial preparations showed no change in maximum force development independent of whether the preparations contained primarily α- or β-cardiac myosin (*6*). Force measurements in larger animals are limited but tend to show that cardiac tissues containing predominantly β-myosin generate about twice the force compared to tissues containing α-myosin (*39*). These data are thus consistent with our findings at the molecular level.

An elegant developmental study of cardiac myosin isoforms by Lompre et al. in 1981 (*8*) showed that the mouse retains predominantly α-myosin throughout its adult lifespan, whereas the amount of α-MHC in animals of increasing size diminishes, until larger mammals contain primarily β-MHC in their left ventricles. This trend is most likely an adaptive response to the power requirements of the heart. The hearts of large animals have a much greater systolic stroke volume than the hearts of smaller animals, and the β-MHC isoform appears to provide the necessary power output (force × velocity) for pumping blood in large mammals. The mouse, to maintain its body temperature, has an unusually high metabolism that leads to a heart rate of ∼600 beats/min compared to ∼70 in humans [cardiac output = (stroke volume/beat) × (beats/minute)]. To optimize its blood circulation, the mouse needs a fast contracting myosin with a high force-generating capacity. The mouse α-MHC isoform appears to satisfy these requirements; it has a velocity of 5−6 μm/s and a force equivalent to that of β-MHC in all species. Thus, evolution has provided the mouse heart with the necessary power output to compensate for its small stroke volume. In larger mammals, the α-MHC isoform is restricted mainly to the atria, where a lower force suffices for its more limited contractile role. From a comparison of the properties of various cardiac myosins, we can conclude that nature has designed cardiac isoforms for optimal myocardial performance in any given species.

ACKNOWLEDGMENT

We thank Dr. P. VanBuren for suggesting the use of α-actinin in the isometric force measurements, Dr. J. J. Sherwood for assistance in chromatographic procedures, and Dr. K. M. Trybus for valuable advice and support in all aspects of this research.

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BI0495329