The kinetics of nucleotide turnover vary considerably among isofoms of vertebrate type II myosin, possibly due to differences in the rate of ADP release from the nucleotide binding pocket. Current ideas about likely mechanisms by which ADP release is regulated have focused on the hyperflexible surface loops of myosin, i.e. loop 1 (ATPase loop) and loop 2 (actin binding loop). In the present study, we investigated the kinetic properties of rat and pig β-myosin heavy chains (β-MHC) in which we have found the sequences of loop 1 (residues 204–216) to be virtually identical, i.e. DQSKKDSQTPKG, with a single conservative substitution (rat E210D pig). Pig myocardium normally expresses 100% β-MHC, whereas rat myocardium was induced to express 100% β-MHC by surgical thyroidectomy and subsequent treatment with propylthiouracil. Slack test measurements at 15 °C yielded unloading shortening velocities of 1.1 ± 0.8 muscle lengths/s in rat skinned ventricular myocytes and 0.35 ± 0.05 muscle lengths/s in pig skinned myocytes. Similarly, solution measurements at the same temperature showed that actin-activated ATPase activity was 2.9-fold greater for rat β-myosin than for pig β-myosin. Stopped-flow methods were then used to assess the rates of acto-myosin dissociation by MgATP both in the presence and absence of MgADP. Although the rates of MgATP-induced dissociation of acto-heavy meromyosin (acto-HMM) were virtually identical for the two myosins, the rate of ADP dissociation was ~3.8-fold faster for rat β-myosin (135 s⁻¹) than for pig β-myosin (35 s⁻¹). ATP cleavage rates were nearly 30% faster for rat β-myosin. Thus, whereas loop 1 appears from other studies to be involved in nucleotide turnover in the pocket, our results show that loop 1 does not account for large differences in turnover kinetics in these two myosin isofoms. Instead, the differences appear to be due to sequence differences in other parts of the MHC backbone.

There is considerable interest in the sequence and subunit composition of myosin isofoms due to the roles that variable expression may have in determining the contractile properties of myocardium and skeletal muscles. Type II myosin molecules are composed of two heavy chains (MHC)¹ (~220 kDa each) and two pairs of light chains (MLCs) designated the essential (~25 kDa) and regulatory light chains (~16 kDa); both the MHCs and MLCs derive from multi-gene families (for review, see Ref. 1). The expression of different myosin subunit isoforms confers a wide range of cross-bridge interaction kinetics in muscles of different types and from different species, variations that are ultimately due to divergence in the primary sequences of the encoding genes.

One feature common to all myosins is the presence of two surface loops (loops 1 and 2) in the MHC subunit, but because of their hyper-flexibility, these loops were not resolved in the x-ray structure of chicken pectoralis myosin (2). Loop 1 is located in the vicinity of the catalytic site, and loop 2 is close to the actin-binding site. The sequences of the loops have been shown to vary significantly between and within classes of myosins (3), and these variations are thought to account for differences in the motile properties of these motor proteins (4).

Studies using chimeric Dictyostelium myosin (5) and chimeric smooth muscle myosin (6) have shown that differences in loop 2 alter the steady state ATPase activity, although it is still not known whether loop 2 is a determinant of Vₘₐₓ, Kₘ, or both. These studies also show that the ATPase activities (in solution) and motor velocities of myosins are not proportional, a finding that is consistent with previous suggestions that actin-activated ATPase and motility have different rate-limiting steps (7) and might therefore be controlled by distinct molecular mechanisms.

The possible roles of loop 1 in conferring the kinetic properties of myosin are not well understood. Spudich (4) proposes that loop 1 regulates the rate of ADP release, which would effectively control the rate of actomyosin dissociation (which occurs almost immediately once ADP dissociates as a result of rapid binding of ATP) and, therefore, the mechanical velocity of the motor protein (8). The idea that loop 1 is a key element in the regulation of the nucleotide turnover rates in myosin has been investigated using gizzard and vascular smooth muscle MHC isofoms, which are spliced products of the same gene and differ in a seven-amino acid insert in loop 1. Initially, Kelley et al. (9) show that gizzard myosin (containing the 7-amino acid insert) had twice the ATPase activity of aorta myosin and translocated F-actin filaments 2.5-fold faster in an in vitro motility assay. Consistent with these results, later studies showed that removal of the insert halved both actin

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¹ The abbreviations used are: MHC, myosin heavy chain; MLC, myosin light chain; AP₅A, P⁵P₅-diadenosine 5’-pentaphosphate; PAGE, polyacrylamide gel electrophoresis; HMM, heavy meromyosin; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; ML, muscle lengths; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine
activated ATPase activity and F-actin sliding velocity (10). These kinetic effects of the loop 1 insert were subsequently related to the rate of ADP release, which was 3-fold faster in gizzard acto-S1 than in myosin without the insert (11). Furthermore, based on results from recombinant myosins with variable loop 1 sequences, Sweeney et al. (11) conclude that the size and the flexibility of loop 1 influence the rate of ADP release and in vitro sliding speed, but that loop 1 is not the only regulator of nucleotide turnover kinetics.

The heterogeneity observed in loop 1 and loop 2 sequences in the myosin super-family (and among isoforms of type II muscle myosins) has been taken as evidence that the loops have significant roles in regulating the kinetic properties of motor proteins (3). Thus, it is tempting to generalize previous conclusions (10, 11) and hypothesize that loop size and flexibility modulate turnover kinetics throughout the class II myosin superfamily. In the present study, we tested this idea by performing a comparative analysis of contractile properties and kinetics of nucleotide turnover in cardiac myosins with identical loop 1 sequences (β-MHC phenotype). The myosins were obtained from a small animal (rat) and a large animal (pig), since any species-dependent kinetic differences would likely be based on differences in animal size (12, 13). Because we find that these myosins with identical loop 1 sequences have dramatically different kinetic properties, we conclude that the sequence of the loop does not confer unique kinetic properties to cardiac myosin nor is the loop the only determinant of myosin turnover kinetics in cardiac β-MHC isoforms.

MATERIALS AND METHODS

Experimental Animals

Rats—Animal usage was conducted under strict guidelines established by the University of Wisconsin Animal Care Committee. All animals were housed in temperature- and light-controlled quarters and were provided with food and water ad libitum. Thyroidectomized female Harlan Sprague-Dawley rats weighing 200–252 g were purchased from Harlan Sprague-Dawley (Madison, WI). Propylthiouracil (12 mg/kg) was administered daily by intraperitoneal injection for 5 weeks to all thyroidectomized rats, since the combination of thyroidectomy and supplemental propylthiouracil administration has been shown to virtually eliminate circulating thyroid hormone (T3 and thyroxine (T4)) (14). Hearts from thyroidectomized rats express virtually 100% β-MHC. Additional rats were made hyperthyroid to obtain hearts expressing 100% α-MHC phenotype. The myosins were obtained from a small animal (rat) and a large animal (pig), since any species-dependent kinetic differences would likely be based on differences in animal size (12, 13). Because we find that these myosins with identical loop 1 sequences have dramatically different kinetic properties, we conclude that the sequence of the loop does not confer unique kinetic properties to cardiac myosin nor is the loop the only determinant of myosin turnover kinetics in cardiac β-MHC isoforms.

Myosin Phenotypes

MHC phenotypes were assessed by SDS-PAGE using conventional methods for the separation of cardiac isoforms (16) and with ultra-sensitive pulse-electrophoresis for SDS gels.2 Phenotypes were confirmed by Western blotting using a monoclonal antibody specific to β-MHC (219–1D1) (18). Characterization of the purity of chymotrypsin-generated HMM was performed on 12–14% separating gels (2% bisacrylamide), and MLC composition was assessed using either 16% glycerol or 14% Tricine separating gels. All gels were silver-stained except for the HMM gels, which were stained with Coomassie Brilliant Blue.

Amino Acid Sequences of Loop 1

Amino acid sequences for the two β-MHC isoforms were predicted from cDNAs for the β-MHCs of rat and pig. RNA was isolated (19), and its quality was assessed by agarose gel electrophoresis. cDNA synthesis was performed with an RNase H-deficient reverse transcriptase (Taqomertm RT, Life Technologies, Inc.) according to the manufacturer’s instructions using an oligo(dt) (20) and another reverse primer (5′-gtgatggcttgagaaacg-3′) that is complementary to exon 36 of all mammalian class II MHCs (20). Characterization of the purity of chymotrypsin-generated HMM was performed using the Platinum DNA polymerase system (Life Technologies), also according to manufacturer’s instructions, using the following primers: reverse for both rabbit and pig (5′-cagctgctgctttctccctggga-3′); forward for rat (5′-gctacattgcttgaaggcc-3′) and forward for pig (5′-aagctgctgaaggggctttc-3′); 5′-ca-gctgctcttcccggagga-3′; 5′-gtgatggcttgagaaacg-3′.

Unloaded Shortening Velocity (V0)

V0 was measured in maximally activated myocytes using the slack test method as described by Strang et al. (15). Once steady tension was achieved in activating solution, the myocyte was slackened by 12–17% of initial length, corresponding to a sarcomere length of 2.25 μm. The time between imposition of a slack step and the redevelopment of force was measured by fitting a horizontal line through the tension base line. The maximum slack imposed was such that sarcomere length did not shorten below 1.90 μm, which is about 0.1 μm greater than the length at which distortion due to mechanical restoring forces within the myocyte is likely to occur (15). Length change (as percent initial length) was plotted versus duration of unloaded shortening (ts), and V0 was determined from the slope of a line fitted to the data by linear regression analysis. Data from a given myocyte were discarded if the regression coefficient was <0.95.

Purification of Myosin and Generation of HMM

To purify myosin, the frozen muscle powder was gradually mixed with a buffer (10.0 mI/g) consisting of 25 mM NaCl, 25 mM EGTA, 50 mM MOPS, 50 mM NaCl, 5.0 mM CaCl2, 118.0 mM NaCl, 2.0 mM KH2PO4, 5.0 mM pyruvate, 11 mM glucose, and 25.0 mM HEPES, pH 7.4). This was followed by successive preparations with a Ca2+-free Ringer’s solution (45 min) and then with a collagenase-containing (1.0 mg/ml) Ringer’s solution (11 min) that also contained hyaluronidase (1.0 mg/ml) and 0.05 mM CaCl2. The ventricles were subsequently minced and incubated for 20 min with the collagenase-containing Ringer’s solution plus 0.25% trypsin. Trypsin inhibitor (10 mg/ml, Sigma) was then added to the solution. Undigested material was removed by filtration using a 300-μm Teflon mesh, and the cells were suspended in Ringer’s solution containing 1.0 mM Ca2+. All solutions used in the isolation procedures were maintained at 37 °C and bubbled with 100% O2. The isolated cells were then skinned for 6 min at 22 °C in a solution containing 1.0 mM free Mg2+, 100 mM KCl, 2.0 mM EGTA, 4.0 mM ATP, 10 mM imidazole, pH 7.0, and 0.3% Triton X-100. The skinned cells were finally washed in skinning solution without Triton X-100 and stored at 0 °C for use on the same day.

2 J. A. A. Sant’Ana Pereira, M. L. Greaser, and R. L. Moss, submitted for publication.
6.0 mM magnesium acetate, 4.0 mM acetic acid, 5.5 mM MgATP, 2 mM DTT, leupeptin (50 μg/ml), pepstatin A (7 μg/ml), and 0.2 mM phenylmethylsulfonyl fluoride at pH 7.1. The samples were stirred on ice for 5 min and then centrifuged at 10,000 × g for 10 min (4 °C) to pellet the myofibrils. After discarding the supernatant, an aliquot of each sample was removed for SDS-PAGE analysis, and the remaining pellet containing the myofibrils was re-suspended in an extraction solution consisting of 0.3 M KCl, 10 mM Na$_3$PO$_4$, 1 mM MgCl$_2$, 5 mM EGTA, 150 mM K$_3$HPO$_4$, 20 mM DTT, leupeptin (50 μg/ml), pepstatin A (7 μg/ml), and 0.2 mM phenylmethylsulfonyl fluoride, pH 6.8. This suspension was stirred constantly on ice for 90 min and then centrifuged for 20 min at 27,000 × g (4 °C) to remove unwanted cellular debris. The supernatant was diluted in 20 volumes of ice-cold water containing 1 mM DTT and allowed to stand for 2 h at 4 °C. The cloudy solution was centrifuged at 10,000 × g for 15 min, and the pellets were then resuspended in solution containing 0.3 M KCl, 10 mM imidazole, 5 mM MgCl$_2$, 20 mM DTT, 10 mM ATP, leupeptin (5 μg/ml), and pepstatin A (0.7 μg/ml), pH 6.8. The sample was gently homogenized and centrifuged in a Beckman Optima TLX Ultrufuge at 480,000 × g for 10 min to pellet-contaminating actin. The supernatant was recovered, diluted with 8 volumes of ice-cold water, and allowed to stand on ice for 3 h. The solution was then centrifuged at 27,000 × g for 10 min. The myosin pellet was re-dissolved in ~5 ml of 0.6 M KCl, 2 mM MgCl$_2$, 5 mM DTT, and 10 mM Tris, pH 7.6. An aliquot was used for determination of protein concentration. The samples were subjected to a myosin digestion of HMM and digestion of HMM was initiated by the addition of 1-chloro-3-tosyl-amido-7-amino-2-heptanone-treated chymotrypsin to 20 μg/ml. The sample was mixed and immediately transferred to a dialysis slide (10,000-Da pore) and dialyzed with constant stirring at 4 °C against 4 liters of 0.6 M KCl, 2 mM MgCl$_2$, 5 mM DTT, and 10 mM Tris, pH 7.6. This procedure was designed to remove contaminant nucleotide from the samples. The myosin digestion was stopped after 7 h by the addition of phenylmethylsulfonyl fluoride to 1 mM. A 120 μM stock of F-actin was dialyzed against 25 mM MOPS, 1 mM EGTA, 1 mM MgCl$_2$, 1 mM DTT, pH 7.4) with one containing 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, or 10.0 mM MgATP (identical salts). To measure the rate of MgADP dissociation from acto-HMM-MgATP, 40 μM or 180 μM MgADP was added to the acto-HMM-MgATP solution before being mixed with the same ATP concentrations as above. Exponential fits to the time course of light scattering were performed using software routines supplied by the stopped flow manufacturer. Typically the rates of 3–6 stopped flow records were averaged to obtain a rate representative of a given MgATP concentration.

**RESULTS**

**Myosin Phenotypes**—Gel analysis using conventional SDS-PAGE and pulse-PAGE showed that the MHC content of myocardium from both pig and hypothyroid rat ventricles were single protein bands with migration mobilities characteristic of β-MHC (Fig. 1A). Western blots confirmed that both pig and hypothyroid rat ventricles expressed 100% β-MHC, i.e. there was no immunoreactivity of tissues with an anti-α MHC monoclonal antibody (results not shown) and positive immunoreactivity with an anti-β MHC monoclonal antibody (Fig. 1B). Euthyroid rats exhibited positive immunoreactivity to both monoclonal antibodies. Pulse-PAGE also showed that the migrations of rat and pig β-MHCs were similar but not identical, suggesting that these two motor proteins have some differences in primary sequence. Samples from hyperthyroid rats exhibited a single protein band corresponding to α-MHC (Fig. 1A).

**Loop Sequences**—The amino acid sequences of loop 1 were obtained from the cDNA sequences for both rat and pig β-MHCs. The sequences were aligned to the numbering of the chicken pectoralis MHC sequence (2) and include the nucleotide binding site (designated the “phosphate loop”) and loop 1 (residues 204–216). The results indicate that these two motor proteins have some differences in primary sequence. Samples from hyperthyroid rats exhibited a single protein band corresponding to α-MHC (Fig. 1A).

**Shorthining Velocities**—Unloaded shortening velocities ($V_0$) were assessed at 15 °C in maximally activated permeabilized myocytes from both pig and hypothryoid rat ventricles (Table 1). The procedure used to attach the myocytes to the apparatus yielded consistently small end compliances; since sarcomere length decreased by 3% or less during the transition from rest to maximal activation, $V_0$ was ~3–fold faster in rat left ventricular myocytes expressing β-MHC (1.1 ± 0.8 ML/s; mean ± S.D., n = 8) than in pig left ventricular myocytes (0.85 ± 0.05 ML/s; mean ± S.D., n = 5). Control experiments on left ventricular myocytes from euthyroid rats showed that $V_0$ in myocytes expressing 100% α-MHC was ~2.3-fold faster (2.5 ± 1.2 ML/s; n = 10) than for rat myocytes expressing 100% β-MHC.

**Actin-activated Myosin ATPase Activity**—The rates of sub-
strate utilization by rat and pig acto-β-HMMs were assessed by measuring actin-activated ATPase activity at low ionic strength and 15 °C. The ATPase rates for both motor proteins were hyperbolic with respect to actin concentration and exhibited saturation kinetics at high actin concentrations, which allowed us to use double-reciprocal plots to estimate both $V_{\text{max}}$ and $K_m$ (Table I). While $V_{\text{max}}$ of the ATPase was 2.9-fold greater in rat than in pig β-HMM, the $K_m$ for the two motor proteins differed by just 1.5-fold.

**Cleavage Rates**—The rates of ATP cleavage by rat and pig β-HMMs were estimated from the time course of protein fluorescence increase observed after mixing with MgATP (25). The measurements were performed at 15 °C, 120 mM ionic strength, and pH 7.4. In these studies we assumed that the reaction mechanism was similar to that deduced by Johnson and Taylor (25) as given by reaction mechanism 1 below

$$
\text{HMM} + \text{ATP} \leftrightarrow \text{M-ATP} \rightarrow \text{M-ATP}^* \rightarrow \text{M-ADP-Pi}** \quad (\text{Eq. 1})
$$

where M is myosin. Step c is a diffusion-limited collision reaction, step i is a fast ($k_i > 1000 \text{ s}^{-1}$) essentially irreversible isomerization, and step h is a readily reversible hydrolysis step where ** indicates an increased protein fluorescence. All the samples exhibited a typical fluorescence enhancement of about 35% upon mixing with MgATP with HMM (Fig. 2A), and the rate of fluorescence increase rose with increases in [MgATP] (Fig. 2A). All traces were well fit with a single exponential, and plots of the observed rates ($k_{\text{obs}}$) versus increasing [MgATP] (Fig. 2A) yielded rates that rose toward an asymptote equal to ($k_h + k_{-h}$) (25, 27). When [ATP] is small, $K_{\text{ATP}} < k_{\text{obs}} < (k_{\text{a,b}} + k_{\text{b,h}})$, so that $k_{\text{obs}} = k_h k_i [\text{ATP}]$. Thus, linear regression analysis of $k_{\text{obs}}$ versus [MgATP] for values less then 50 μM yielded a slope corresponding to $K_h k_i$. These plots indicated that $K_h k_i$ for porcine cardiac β-HMM was $3.3 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$, and for rat β-HMM was $6.0 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$. These values are in good agreement with earlier work using cardiac S-1 (27). The value of ($k_{\text{a,b}} + k_{\text{b,h}}$) was approximated by progressively increasing the [MgATP] until $k_{\text{obs}}$ no longer changed. The values of $k_{\text{obs}}$ at 1 and 2.5 mM MgATP were not significantly different in either of the HMMs tested so that ($k_{\text{a,b}} + k_{\text{b,h}}$) was computed from the average $k_{\text{obs}}$ at these two concentrations. Thus ($k_{\text{a,b}} + k_{\text{b,h}}$) for rat and pig β-HMM were $63.5 \pm 1.4 \text{ s}^{-1}$ and $43.5 \pm 0.7 \text{ s}^{-1}$ ($n = 8$), respectively. These results are consistent with the remarkably similar structures of the two myosins and with results from earlier studies (27).

**Kinetics of Actomyosin-HMM Dissociation**—Estimates of the kinetics of ATP binding to and ADP dissociation from rat and pig β cardiac acto-HMMs were obtained by measuring the ATP-dependent rate of acto-HMM dissociation in the presence and absence of added ADP. The use of ADP as a competitive inhibitor of ATP binding at the active site allows estimation of ADP off rates (26). The reaction for the increase in light scattering is given in mechanism 2 below

$$
\text{A} \leftrightarrow \text{ADP} + \text{AM} \leftrightarrow \text{ATP} \leftrightarrow \text{A + M-ATP} \quad (\text{Eq. 2})
$$

where A is actin, M is myosin, and AM is actomyosin. In the absence of ADP, only steps 1 and 2 occur when acto-HMM is rapidly mixed with MgATP. Fig. 2B shows the decrease in light scattering upon mixing acto-HMM with acto-HMM (15 °C and 120 mM ionic strength), which effectively reports dissociation of acto-HMM. The data for rat acto-HMM were well fit using a single exponential, which was the case for 8 of 17 different conditions measured. When double exponential fits were made, the fast phase had a similar rate to that fitted by a single exponential, and the second phase amplitude averaged 7.3% that of the fast phase. The data for pig acto-HMM dissociation were also well fit (mean $r^2 = 0.983$), with a single exponential in 13 of the 31 conditions studied. However, a double exponential yielded a better fit in the remainder of the experiments. In these cases the amplitude of the second phase averaged 26.0 ± 3.0% (±S.E.) of the total change in light scattering, and its rate was << $s^{-1}$, whereas that of the fast phase was 2–3 times faster. As [MgATP] increased, the slower second phase rate increased relatively little, whereas the first phase became much faster. As [MgATP] increased, the observed amplitude of the first phase declined because an increasing fraction of the phase occurred within the dead time of the stopped-flow device. Thus, determinations of observed rates greater than 500–600 s$^{-1}$ were less accurate, as fewer data points were available for fitting. Similar two-phased behavior has previously been ob-

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Fig. 1. Characterization of MHC phenotypes of rat and pig left ventricular myocardium. A, upper panel, standard 12% SDS-PAGE. Lane 1, MHC composition in euthyroid rats (fastest migrating band corresponds to β-MHC). Lane 2, MHC composition in hypothyroid rats. Lane 3, MHC composition in euthyroid pig. Middle panel, pulse-PAGE in 12% SDS gels, which were run for 78 h. Lane 1, MHC composition in euthyroid rats (fastest migrating band corresponds to β-MHC and the two upper bands correspond to two α-MHC isoforms). Lane 2, MHC composition in hypothyroid rat. Lane 3, MHC composition in euthyroid pig. Lower panel, Western blot analysis of MHC composition using anti-β MHC monoclonal antibody. Lane 2, hypothyroid rat. Lane 3, euthyroid pig. Both samples exhibit positive immunoreactivity. B, resolution of the MLC composition of left ventricular myocardium of different species in 14% Tricine SDS-PAGE. From left to right: Pg, pig; Dg, dog; Rb, rabbit; Ms, mouse; Rt, rat.
observed in cardiac myosin, and the slower, less ATP-sensitive rate has been attributed to denatured protein (27). However, we conclude that the slower phase was produced by ADP contamination of our pig acto-HMM preparation. This conclusion follows from our observation that treatment of a pig acto-HMM preparation with 4 units of apyrase/ml acto-HMM for 2 h before stopped-flow experimentation abolished the second slow phase.

**Fig. 2.** Stopped flow measurements of HMM fluorescence increase, the dissociation of actomyosin-HMM, and the dissociation of actomyosin-HMM-ADP by MgATP. Panel A shows the time course of the intrinsic fluorescence increase marking the ATP hydrolysis by rat (a) and pig (b) HMM at 100 μM MgATP. The rate of the fluorescence increase in trace a is 43.0 ± 0.4 s⁻¹ and in trace b is 35.9 ± 0.3 s⁻¹. Panel B shows the decrease in light scattering by acto-HMM in rat (a) and pig (b) acto HMM in 250 μM MgATP. Trace a was fitted by a single exponential whose rate was 330.7 ± 7.7 s⁻¹ (amplitude = 3.17 V), and trace b was fitted by a double exponential whose rates were 392.8 ± 25.7 s⁻¹ (amplitude = 4.31 V) and 8.2 ± 4.9 s⁻¹ (amplitude = 2.5 V). Panel C shows the dissociation of rat (a) and pig (b) acto-HMM-ADP in the presence of 2.5 mM MgATP. Traces a and b were fitted with single exponentials with rates of 135.5 ± 0.4 s⁻¹ and 38.7 ± 0.3 s⁻¹, respectively. In each case, r² > 0.97. Each reaction time course is the average of 3–4 separate recordings. The solid smooth lines are exponential fits to the data with the indicated rates.

**Fig. 3.** ATP dependence of the rates of intrinsic protein fluorescence increase, acto-HMM dissociation, and acto-HMM-ADP dissociation. Panel A shows the increase in intrinsic fluorescence for rat (a) and pig (b) β-MHC. Panel B is the rate of acto-HMM dissociation in the absence of ADP for rat (a) and pig (b) acto-HMM. Panel C is the rate of acto-HMM dissociation in the presence of MgADP for rat (a) and pig (b) acto-β-HMM. In the experiments using rat β-HMM, the [MgADP] was 90 μM and for pig was β-HMM was 25 μM (20 μM added and 5 μM contamination). The solid lines are fits to the data described in the text. In each case, filled circles represent data from experiments using rat β-HMM and filled circles for pig β-HMM. The error bars represent ± 1 S.E. but did not change the fast ATP-dependent rate. Thus, this contamination does not materially affect the conclusions from these experiments.

Fig. 3B is a plot of the rate of AM dissociation as a function of [MgATP]. As the rates of dissociation for the rat and pig preparations did not appear to differ significantly, the data were fit by a single line. The solid line is a fit to the data of the following form.

$$k_{obs} = k_d K_f [\text{ATP}] / (K_f [\text{ATP}] + 1)$$

(Eq. 3)

This fit yielded $k_d K_f$ of 1.7 ± 0.24 × 10⁶ M⁻¹ s⁻¹ and a $K_f$ value of 904 ± 444 M⁻¹. These results imply that $k_d$ is 1880 s⁻¹ for rat

**TABLE I**

Measured kinetic constants

| Parameter | Rat | Pig |
|-----------|-----|-----|
| Unloaded shortening velocity (ML s⁻¹) | 1.10 ± 0.08 | 0.35 ± 0.05 |
| Acto-S1 ATPase | | |
| $V_{max}$ (s⁻¹) | 0.23 ± 0.04 | 0.08 ± 0.01 |
| $K_m$ | 13.5 ± 0.6 | 8.6 ± 0.7 |
| ATP binding to HMM, $K_i k_1$ (s⁻¹) | 6.09 ± 0.03 × 10⁵ | 2.70 ± 0.01 × 10⁵ |
| ATP cleavage by HMM, $k_{-1} + k_{-2}$ (s⁻¹) | 63.5 ± 1.4 | 43.5 ± 0.7 |
| ATP induced acto-HMM dissociation | | |
| $K_0$ (M⁻¹) | 904 ± 444 | 904 ± 444 |
| $k_2$ (s⁻¹) | 1.7 ± 0.2 × 10⁶ | 1.7 ± 0.2 × 10⁶ |
| $K_i k_2$ (s⁻¹) | 1.98 × 10⁷ | 1.98 × 10⁷ |
| ADP binding and release from acto-HMM | | |
| $k_{-e}$ (s⁻¹) | 135.4 ± 5.8 | 35.4 ± 2.3 |
| $k_{-e} K_f$ (s⁻¹) | 3.0 × 10⁶ | 3.0 × 10⁶ |
| $k_{-e} K_f$ (s⁻¹) | 1.7 × 10⁶ | 1.7 × 10⁶ |

FIG. 2. Stopped flow measurements of HMM fluorescent increase, the dissociation of actomyosin-HMM, and the dissociation of actomyosin-HMM-ADP by MgATP. Panel A shows the time course of the intrinsic fluorescence increase marking the ATP hydrolysis by rat (a) and pig (b) HMM at 100 μM MgATP. The rate of the fluorescence increase in trace a is 43.0 ± 0.4 s⁻¹ and in trace b is 35.9 ± 0.3 s⁻¹. Panel B shows the decrease in light scattering by acto-HMM in rat (a) and pig (b) acto HMM in 250 μM MgATP. Trace a was fitted by a single exponential whose rate was 330.7 ± 7.7 s⁻¹ (amplitude = 3.17 V), and trace b was fitted by a double exponential whose rates were 392.8 ± 25.7 s⁻¹ (amplitude = 4.31 V) and 8.2 ± 4.9 s⁻¹ (amplitude = 2.5 V). Panel C shows the dissociation of rat (a) and pig (b) acto-HMM-ADP in the presence of 2.5 mM MgATP. Traces a and b were fitted with single exponentials with rates of 135.5 ± 0.4 s⁻¹ and 38.7 ± 0.3 s⁻¹, respectively. In each case, r² > 0.97. Each reaction time course is the average of 3–4 separate recordings. The solid smooth lines are exponential fits to the data with the indicated rates.
and porcine β-HMMs. The error in $K_b$ is large because accurate measurements at higher [ATP] were not possible.

If ADP is added to an acto-HMM solution before mixing with MgATP, the reaction shown in mechanism 2 (Equation 2) occurs. If the release of ADP from acto-HMM, $k_{-5}$, is much slower than the rate of acto-HMM dissociation by ATP, i.e., $k_{-5}K_b[MgATP]$, then at high concentrations of MgATP the rate of decrease in light scattering will be limited by and equal to $k_{-5}$. At small concentrations of MgATP (where $k_{-5} > K_b[MgATP]$), MgADP reduces the $k_{obs}$ by competing with MgATP for binding to AM and so decreases the fraction of A-HMM available to pass through steps 1 and 2. Thus, at low [MgATP], $k_{obs}$ will be small and will rise toward $k_{-5}$ as [MgATP] is increased. The rate of acto-HMM dissociation was accurately fitted by a single rate of dissociation for both pig and rat acto-HMM-ADP because the amount of ADP added to the acto-HMM in each case was selected to produce a large fraction of actomyosin as acto-HMM-MgADP before mixing. In addition, there was significant ADP contamination of the pig acto-HMM, which resulted from its 4-fold higher affinity for MgADP as compared with rat acto-HMM. To obtain an estimate of $k_{-5}$, we used the value of $K_b k_{-2}$ from the data in Fig. 3B and the asymptote in Fig. 3C, a for $k_{-5}$ in Equation 2. Using $K_b k_{-2}$ and $k_{-5}$, we computed the rates of rat acto-HMM-MgADP dissociation using simulations of Equation 2 and found that the rates observed at different [MgATP] were predicted if $k_{-5} = 3 \times 10^6$ $M^{-1} s^{-1}$ (the solid lines in Fig. 3C, a are the predicted rates). We then assumed the same $k_{-5}$ for pig acto-HMM and found that the predicted dissociation rates were accurately predicted by the equation (see solid line in Fig. 3C, b) if we assumed that there was 10 $\mu$m contaminating Mg-ADP in the pig acto-HMM before the addition of MgADP. The results of the fitting process are presented in Table I. Because the length and sequence of loop 1 are essentially identical for the two species and the rate of ADP release from acto-rat β-HMM is about four times faster than that for acto-pig β-HMM, we conclude that the rate is not controlled by loop 1. Furthermore, the larger rate of ADP release from acto-rat-β-HMM is consistent with the differences observed in unloaded shortening velocity measurements in skinned myocardial preparations (Table I).

DISCUSSION

Previous studies of the roles of loop 1 in smooth muscle myosin isoforms showed that the peptide sequence of loop 1 is an important modulator of kinetic properties (9–11). If the peptide sequence of loop 1 is the primary determinant of the kinetic properties of muscle myosin isoforms, as has been suggested by Spudich (4), myosins having identical loop sequences would be expected to exhibit similar kinetic properties. In this study, we tested this hypothesis using pig and rat cardiac β myosins, which exhibit no differences in loop 1 sequence (see “Results”). Contrary to expectations, our results show that kinetic properties differ markedly between these two cardiac myosins.

Several biochemical and physiological variables were used in this study to assess differences in kinetics between rat and pig myosins containing β-MHC. Unloaded shortening velocity, which varies with the rate of cross-bridge detachment (28), was used as an indirect assay of ADP off rates (for review, see Ref. 29). Rat myocytes expressing α-MHC were used as controls and exhibited shortening velocities that were 2.2-fold faster than rat myocytes expressing β-MHC, which is consistent with previous results from cardiac myocytes (15) and from purified myosin in an in vitro motility assay (30). Remarkably, there was a 3-fold difference in $V_o$ between rat β-MHC and pig β-MHC myocytes measured under identical conditions (Table I). Although these results strongly suggest that there are marked differences in kinetics between the two β-MHC motor proteins studied here, such results are not entirely definitive because they were obtained from preparations with intact myofilaments, which in rat and pig myocytes contain different regulatory proteins. Consequently, more direct kinetic measurements were performed using HMM generated from purified myosins from both pig and hypothyroid rat myocardium.

Stopped-flow measurements showed that the ADP off rate differed ~4-fold between rat and pig β-HMM myosins. These results indicate that there is good proportionality between mechanical measurements of $V_o$ and the rates of ADP dissociation, which is consistent with the idea that ADP off rate is the primary determinant of $V_o$ (8). It seems likely that the variations in β myosin heavy chain account for the differences in cross-bridge kinetics observed in this study. A potential criticism of this conclusion is that $V_o$ is also modulated by MLC content, at least in skeletal muscle, and it is possible that pig and rat myosins do not have identical MLC content. For example, specific depression of shortening velocity (or F-actin sliding velocity in vitro) has been reported in MLC-deficient myosins (31, 32) and in LC-reconstituted skeletal muscle myosins expressing greater amounts of MLC 1f than MLC 3f (33, 34). It is unlikely that any of the myosins in our studies was LC-deficient, since MHC:MLC stoichiometry was similar in all of our SDS gel samples (results not shown). Another possibility that is difficult to eliminate completely is that there are differences in the sequences of rat and pig cardiac MLCs. In this regard, the sequences for pig cardiac MLCs are still unknown, making it impossible to identify amino acid substitutions that might have significant effects on function. At a superficial level, both pig and rat left ventricular myocardium express MLC 1v and MLC 2v isoforms that migrate similarly on SDS-gels, suggesting that the isoforms are likely to be highly homologous in the two species (Fig. 1B). To date, investigations of possible effects of altered MLC composition in the mammalian heart has been done in only a few studies. However, mouse transgenic studies appear to indicate that remodeling the MLC isoform expression in the heart, at least for the regulatory MLC, does not produce large changes in kinetics. For example, partial replacement of MLC 2v with skeletal fast isoform (MLC 2f) does not alter $V_o$ in myocardium, even though it reduces ATPase activity by about 20% (35). In the present study, we observed near proportionality between $V_o$ and the rate of ADP dissociation as well as with the rate of actin-activated ATPase for the β-MHC motor proteins studied here. Although it is possible that differences in MLCs influenced the kinetic properties of these motor proteins, it is improbable that such differences could account for the 3–4-fold differences in kinetic properties observed between the rat and pig isoforms of β-MHC.

There have been some inconsistencies in previous studies regarding possible roles of loop 1 in the actin-activated ATPase activity of myosin. Results from recombinant Dictyostelium myosins suggested that $V_{max}$ is determined by the sequence of loop 2 (5). Subsequent studies on recombinant smooth muscle myosins showed that $K_m$ (not $V_{max}$) is determined by loop 2 (6) and that $V_{max}$ is influenced by alterations in loop 1 (10, 11). In view of these earlier results, the sequences of loop 1 in the myosins used in the present study were also assessed. Our results show that the peptide sequences of loop 1 were identical in rat and pig β-MHCs, and yet $V_{max}$ was 2.9-fold faster for rat β-HMM than for the pig. Obviously, loop 1 cannot account for the kinetic differences between these two heavy chains. Similarly, it must be concluded that loop 1 does not directly regulate mechanical or biochemical $V_{max}$ in cardiac myosins. Furthermore, the $K_m$ of the ATPase differed for the two β-MHCs, although the difference (1.5-fold) was approximately half that observed for $V_{max}$. This disparity in degree of difference for these variables in the two β-MHCs suggests that different
domains of the molecule are involved in setting these variables. In this regard, it should be noted that the loop 2 sequences of rat β-MHC and pig β-MHC exhibit one single nonconserved substitution, i.e. rat A631T pig, according to the chicken pectoralis numbering scheme (2).

Results obtained in this study also indicated that the rate of ATP cleavage ($k_{-a} + k_{-c}$) was 30% faster in rat (−64 s−1) than in pig (−44 s−1) β-HMM myosins. Similarly, the binding constants ($K_{k_c}$) for ATP were somewhat greater for rat (6.00 ± 0.33 × 105 M−1 s−1) than for pig (2.70 ± 0.01 × 105 M−1 s−1), suggesting that these properties of myosin are not directly related to the sequence of loop 1.

Overall, the results of this study support the interpretation that the peptide sequence of loop 1 per se does not account for the distinct kinetic properties of β-MHCs from different mammalian species and do not support the hypothesis proposed by Spudich (4) concerning possible roles of loop 1. Thus, other regions or domains of the myosin molecule must ultimately govern the kinetic properties studied here. Amino acid sequence comparisons between rat and pig β-MHCs using both published sequences (GenBank™ accession numbers AA37320 for pig β-MHC and S06006 for rat β-MHC) and our own results indicate that there are regions of divergence, localized in three clusters: 1) 5 substitutions between residues 323 and 351, which forms an α-helix close to the catalytic domain, in proximity to switch 1 and switch 2 and extending to the actin binding site; 2) 3 substitutions between residues 423 and 432, located at the binding interface with actin; and 3) 4 substitutions between 575 and 612, also at the myosin-actin binding interface. Amino acid substitutions in each of these regions have been associated with familial hypertrophic cardiomyopathy, and it seems likely that the kinetic differences observed in the present study are due to one or more of these sequence differences. Our observations are consistent with the previous interpretation (11) that loop 1 is not the primary determinant of kinetic differences between myosin isoforms.

Certainly, our results do not eliminate the possibility that loop 1 is involved in regulation of myosin kinetics. From the results of Sweeney et al. (11), in which removal of the loop virtually stopped ADP release from the pocket, it appears that the loop is at least necessary for nucleotide turnover. Thus, it is reasonable to speculate about the role of loop 1 in myosin function.

Much of our understanding of kinetic mechanisms in vertebrate myosins has come from studies in smooth muscle myosins; however, conclusions from smooth muscle myosins might not be easily extrapolated to cardiac myosins. In this regard, smooth muscle myosin isoforms are spliced products of the same gene and exhibit (at least in their HMM subunits) variations only in loop 1, whereas in cardiac β myosins, the sequence of the loop 1 is identical but the backbone varies. Because some of the substitutions in these β myosins are close to the active site, it is reasonable to assume that their kinetics are primarily determined by residues in the catalytic domain. In such a case, the role of loop 1 would be to somehow influence, either to hinder or promote, catalytic events within the binding pocket. For this to occur, loop 1 would have to interact with sites in the backbone of the molecule that directly govern the kinetic properties of the motor protein.

It is well recognized that tryptic digestion of HMM cleaves loop 1. However, the presence of nucleotide either in solution or cross-linked to the active site of the protein offers protection against proteolysis of the loop (36). This result suggests that loop 1 can assume two different conformations, one in the absence and the other in the presence of nucleotide in the active site (the latter making the cleavage site less susceptible to proteolysis). All cardiac, skeletal, and smooth muscle MHCs express charged consensus motifs at the NH2-terminal ends of their respective loops (chicken pectoralis numbering): cardiac 240DSKKKD216 skeletal 240EKKE/E(D)209 smooth 260SHGGKD209. It is therefore conceivable that such residues would mediate an interaction between loop 1 and another region of the myosin backbone. Although we have yet to produce recombinant proteins to test this idea, such a model would explain why smooth muscle constructs (in which the only difference is the sequence of loop 1) expressing a variety of foreign loops exhibited precisely the same ATPase $V_{max}$ as the loopless constructs (11). This model also provides an explanation for the positive correlation between the ADP off rates and loop size among recombinants (11); increasing the size of the loop might increase the probability of interaction, if only to a small degree.

Thus, we do not believe that such a model conflicts with the results of Sweeney et al. (11) regarding their myosin constructs having loops with lysine removal or charge reversal, since these mutants retained charged residues in the original positions of their consensus motifs. Finally, it should be taken into account that loop 1 may not govern, directly or indirectly, the ADP off rates of myosin (17) but, rather, other molecular steps.

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