Alternative Exon-encoded Regions of Drosophila Myosin Heavy Chain Modulate ATPase Rates and Actin Sliding Velocity*

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To investigate the molecular functions of the regions encoded by alternative exons from the single Drosophila myosin heavy chain gene, we made the first kinetic measurements of two muscle myosin isoforms that differ in all alternative regions. Myosin was purified from the indirect flight muscles of wild-type and transgenic flies expressing a major embryonic isoform. The in vitro actin sliding velocity on the flight muscle isoform (6.4 μm/s) at 22 °C is among the fastest reported for a type II myosin and was 9-fold faster than with the embryonic isoform. With smooth muscle tropomyosin bound to actin, the actin sliding velocity on the embryonic isoform increased 6-fold, whereas that on the flight muscle myosin slightly decreased. No difference in the step sizes of Drosophila and rabbit skeletal myosins were found using optical tweezers, suggesting that the slower in vitro velocity with the embryonic isoform is due to altered kinetics. Basal ATPase rates for flight muscle myosin are higher than those of embryonic and rabbit myosin. These differences explain why the embryonic myosin cannot functionally substitute in vivo for the native flight muscle isoform, and demonstrate that one or more of the five myosin heavy chain alternative exons must influence Drosophila myosin kinetics.

The functional properties of muscle myosins have been investigated in many systems. Developmental- and tissue-specific isoforms have distinct ATPase rates and velocities that are the primary determinants of muscle contractile properties (1). However, it is not well understood how functional properties of myosin are determined by variations in myosin heavy chain (MHC) sequence and structure (2, 3).

Genetic engineering, sequence comparisons, in vitro motility assays, and crystallographic structures have facilitated recent investigations into MHC structural regions that might influence function properties (3). For example, the two flexible loops (25/50-kDa, loop 1; 50/20-kDa, loop 2) in the chicken skeletal and Dictyostelium myosin heads, unresolved in the atomic structures, have been examined as possible determinants of functional variation between isoforms (4–6). Substituting different loop 1s with varying charge and length affected ADP release rate, ATPase rate, and in vitro actin sliding velocity (4). However, these changed properties did not correlate well with the relative speed of the native myosin from which the loop was derived, suggesting that additional regions need to be altered in concert to give the unique kinetic and velocity characteristics of a specific isoform.

Investigations using species-specific myosins differing only in one or a few regions result in better insights. In phasic smooth muscle myosin, seven additional amino acids near the nucleotide binding site increase actin-activated ATPase rate and in vitro motility velocities compared with the tonic smooth muscle isoform (7, 8). Similarly, scallop catch and phasic muscle isoforms have different motility and ATPase rates (9).

The Drosophila system has the potential to provide even greater insights. Fifteen different MHC isoforms, arising from alternative splicing of mRNA transcripts from the single Mhc gene (10, 11), have been found so far in a wide variety of muscle types, ranging from the supercontractile larval muscles to the highly ordered, asynchronous indirect flight muscles (IFMs) (12–15). Four of the five alternatively spliced exon sets occur in the myosin head coding region and have been proposed to determine functional differences between isoforms (Fig. 1) (16). The variety of myosins normally expressed and the ease of creating transgenic flies make the Drosophila Mhc gene a powerful system for studying the relationships between myosin sequence and in vivo functional variation (17). However, to take full advantage of this system, it is necessary to develop in vitro assays to determine the different properties of Drosophila myosins. As the IFMs constitute a major part of the adult fly (approximately 20% of body weight), myosin isolation from these muscles following dissection is relatively straightforward compared with attempting recovery from other muscle types.

Recently, a major embryonic isoform (Emb) was transiently expressed in a myosin null background (18) such that every muscle type expressed only the Emb isoform. Although the transgenic flies were viable, they exhibited a flightless phenotype and were severely impaired in jumping and walking abilities. In addition, the IFMs showed severe myofibrillar degeneration soon after the time at which flight normally commences. Clearly, this embryonic isoform cannot substitute functionally for the IFM isoform. The embryonic isoform differs
from that of the IFM at all alternatively spliced regions; therefore, one or more of the alternative exons must be responsible for the functional differences between these isoforms.

We report on the purification and functional characterization of Drosophila IFM and modified embryonic (Emb<sup>18</sup>) myosins. We present the first optical trap measurements of Drosophila myosin step size, attachment lifetimes, in vitro motility velocities, and isoform-specific ATPase measurements. The results demonstrate that alternatively spliced exon regions of MHC dramatically influence cross-bridge cycle kinetics and set the stage for the evaluation of chimeric MHC constructs to pinpoint functional variations to particular polypeptide sequences encoded by the alternative exons.

### EXPERIMENTAL PROCEDURES

**Myosin Isolation**—Myosin was prepared from the dorsolongitudinal IFMs (DLMs) of 100–120 wild-type (for muscle genes; the flies carry the yellow body color and white eye mutations) or transgenic Emb<sup>18</sup> flies. Emb<sup>18</sup> myosin (hereafter referred to as Emb<sup>18</sup>) was expressed transgenically following P element-mediated germline transformation of a modified version of a major embryonic isoform cDNA (17). Emb<sup>18</sup> was modified from a native embryonic myosin to have the shorter adult tailpiece encoded by exon 18. The adult tailpiece enhances myosin accumulation in the adult IFMs (17). By expressing Emb<sup>18</sup> in a Mhc<sup>10</sup> background (null for myosin in flight and jump muscle), the Emb<sup>18</sup> isoform could be isolated from the IFM without contamination by other myosin isoforms. However, it had the IFM-specific essential myosin light chain isoform. Thus, the only differences between the isolated myosin isoforms were located in head regions encoded by exons 3, 7, 9, and 11 (Fig. 1), and the rod hinge region, encoded by exon 15.

DLMs were dissected at 4 °C from split thoraces in York Modified Glycerol (YMG: 20 mM potassium phosphate, pH 7.0, 2 mM MgCl<sub>2</sub>, 1 mM EGTA, 8 mM DTG, 2% (v/v) Triton X-100, and 50% (v/v) glycerol) (19) without Triton X-100, but with the addition of a protease inhibitor mixture (Complete, Roche Molecular Biochemicals). A protease inhibitor mixture was also included in all subsequent myosin extraction solutions. IFMs were suspended in YMG (with Triton X-100), incubated for 30 min, centrifuged (8,500 × g), and washed in YMG without Triton X-100 and glycerol. Myosin was extracted into three volumes (55 µl) of 1.0 M KCl, 0.15 M potassium phosphate, pH 6.8, 10 mM sodium pyrophosphate, 5 mM MgCl<sub>2</sub>, 0.5 mM EGTA, and 8 mM DTG for 10 min and centrifuged at 8,500 × g for 5 min. The proteins in this initial extraction are shown in lane 2 (Fig. 2A, lane 2). The pellet was sometimes used to make acetic acid powder for actin isolation (see below). The extracted myosin was precipitated by decreasing KCl to 40 mM and incubating for 16 h (overnight). Following a 15-min centrifugation in a Beckman TL-100.3 rotor at 100,000 × g, the pellet (Fig. 2A, lane 3) was dissolved in an equal volume (18 µl) of wash B (2.4 mM KCl, 100 mM histidine, pH 6.8, 0.5 mM EGTA, 8 mM DTG) (20). Myosin that did not dissociate from actin (shown in Fig. 2A, lane 2) was precipitated by slowly adding water until the KCl concentration was decreased to 0.3 M and then centrifuged at 60,000 × g for 30 min. The supernatant was removed and further diluted until the KCl concentration reached 30 mM and centrifuged at 100,000 × g for 30 min. The final pellet was resuspended in 30 µl of myosin storage buffer (0.5 mM KCl, 20 mM MOPS, pH 7.0, 2 mM MgCl<sub>2</sub>, and 5 mM DTG).

**Drosophila** myosin preparations were quantified by their absorbance at 280 nm using an absorbance coefficient of 0.53 cm<sup>−1</sup> for 1 mg·ml<sup>−1</sup> (21). The Bradford assay (Coomassie Plus-200, Pierce) and semiquantitative SDS-PAGE gave similar results when rabbit myosin was used as the protein standard. The Bradford assay (Coomassie Plus-200, Pierce) and semiquantitative SDS-PAGE gave similar results when rabbit myosin was used as the protein standard.

**Actin Isolation**—Actin was isolated from dissected DLMs as described by Razzag et al. (22). Purified F-actin was centrifuged at 150,000 × g for 1.5 h, and resuspended in actin buffer (25 mM imidazole, pH 7.4, 25 mM KCl, 4 mM MgCl<sub>2</sub>, 1 mM EGTA, and 1 mM DTG) (23). Fig. 2C shows the purity of the actin preparation. Actin was quantified by subtracting absorbance at 310 nm from that at 290 nm and dividing by an absorbance coefficient of 0.62 cm<sup>−1</sup> for 1 mg·ml<sup>−1</sup> (24). For the motility assay, actin was labeled with rhodamine-phalloidin as described by Kron et al. (23).

**ATPase Assays**—The Ca-ATPase activity of myosin was measured using a modification of the method described by Pullman (25). Activity was determined in a 0.5-ml reaction mixture containing 10 mM imidazole, pH 6.0, 0.1 M KCl, 10 mM CaCl<sub>2</sub>, and 1 mM γ-[<sup>32</sup>P]ATP (500–800 cpm·mmol<sup>−1</sup>·min<sup>−1</sup>) with 2–5 µg of protein. The reaction was initiated by ATP addition. After incubating for 15 min at 22–23 °C, the reaction was stopped by addition of 0.1 ml of 1.8 M HClO<sub>4</sub>. Following centrifugation, aliquots of the supernatant were added to 0.5 ml of 5% ammonium molybdate, 2 ml of 1.25 M HClO<sub>4</sub>, and 2.5 ml of isobutanol-benzene (1:1). After vigorous mixing and phase separation, 1 ml of the organic phase containing [32P] was assayed by Cerenkov counting. We found that the Ca-ATPase activity at pH 6 was approximately twice that obtained at pH 7.4 (data not shown), similar to previous reports for whole thorax myosin (26). Thus, we measured ATPase activity at pH 6.0 to maximize ATPase rates.

For determination of the K<sub>m</sub> of myosin Ca-ATPase activity, ATP was varied from 5 to 300 µM for IFM myosin and from 2 to 40 µM for Emb<sup>18</sup> myosin. Specific radioactivity of [32P]ATP was increased to 5,000–8,000 cpm·mmol<sup>−1</sup>. The reaction time was reduced to 3 min to ensure that initial rates were determined.

Mg-ATPase activity was determined in 0.5 ml of solution containing 10 mM imidazole, pH 6.0, 20 mM KCl, 2 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, and 1 mM ATP with 2–5 µg of protein for 30 min at 22–23 °C, and processed as described above for Ca-ATPase.

**Sliding Filament Assay**—F-actin in vitro motility assays were conducted similarly to Kron et al. (22), except for the following modifications. 1) The nitrocellulose-coated coverslip of the flow cell was blocked by incubating with assay buffer plus BSA (AB/BSA) prior to myosin addition at 0.5 mg·ml<sup>−1</sup>. 2) After myosin addition, dead heads (myosin that binds irreversibly to actin) were blocked by adding 5 µM phalloidin-stabilized IFM actin followed by AB plus ATP, 0.4% methylcellulose, and oxygen scavengers (27). After a 10-min incubation, ATP was washed out of the flow cell by several additions of AB/BSA. Labeled actin was added and the remainder of the protocol was as described by Kron et al. (23). This method was very effective, as continuous movement of actin filaments would last ~10 min in unblocked cells before the majority of filaments were arrested by dead heads, while in cells employing dead head blocking, most filaments were still moving after 30 min. Filament movement in unblocked cells was used as an indicator of...
myosin quality. If continuous movement by the majority of actin filaments did not last for ~10 min, the myosin preparation was not used for ATPase measurements. 3) The final activating motility solution contained 0.4% methylcellulose and no KCl.

In some experiments, smooth muscle tropomyosin (smTM) (chicken gizzard, Sigma) was bound to Drosophila IFM actin by mixing at a 1:1 molar ratio. Centrifugation and one-dimensional SDS-PAGE electrophoresis of the pellet and supernatant confirmed the binding of smTM to Drosophila F-actin (data not shown). smTM (100 nM) was included in all solutions added to the flow cell after actin addition to prevent smTM dissociation from actin (28).

The assays were conducted at 22–23 °C, and filament movement was recorded on videotape. Video sequences were captured at 10 frames/s using an optical tweezers transducer in the “three-bead” configuration (30–32). Two light chains and light chains on SDS-PAGE gels (Fig. 2B). There are multiple phosphorylated versions of the RLC (~14 isoelectric variants), which result in two bands, 30 and 33 kDa, on the gel (33). C, lane 1 is IFM actin and arthrin, and lane 2 is rabbit actin. Arthrin is the ubiquinated form of actin found only in the IFM (63).

**RESULTS**

**Protein Isolation**—The Drosophila myosin isolation method yielded an enzymatically active myosin capable of moving actin filaments in a smooth and continuous manner. The IFM myosin yield was 300–400 μg (2.5–3.3 μg/fly) and was at least 90% pure, as judged from the proportion of heavy chain (200 kDa) and light chains on SDS-PAGE gels (Fig. 2B). Two light chains associate with Drosophila MHC: the essential light chain and light chains on SDS-PAGE gels (Fig. 2B).

**Histograms of the displacement events were fitted with a Gaussian function, allowing hundreds of displacement events (for details, see Refs. 31 and 32).**

The size of the working strokes of the two Drosophila myosins interacting with rabbit or Drosophila IFM actin were determined by analyzing hundreds of displacement events (for details, see Refs. 31 and 32). Histograms of the displacement events were fitted with a Gaussian distribution whose midpoint was shifted from zero, reflecting the size of the working stroke, and whose width was determined by the trap stiffness (31).

**TABLE I**

ATPase activities

| Isoform  | Ca-ATPase $K_m$ (ATP)$^1$ | Mg-ATPase $K_m$ (ATP)$^1$ | Mg-ATPase $v^{-1}$ | Mg-ATPase $v^{-1}$ |
|---------|--------------------------|--------------------------|-------------------|-------------------|
| IFM     | 8.3 ± 0.3 (4)            | 20.8 ± 1.6 (3)           | 0.11 ± 0.01 (4)   | 0.11 ± 0.01 (4)   |
| Emb$^{18}$ | 3.15 ± 0.4 (3)$^b$       | 4.4 ± 0.8 (3)$^b$        | 0.022 ± 0.002 (3)$^b$ | 0.022 ± 0.002 (3)$^b$ |

$^a$Determined from Ca-ATPase experiments.

$^b$Statistically different from IFM, by Student’s t test ($p < 0.001$).
The yield of IFM actin was ~0.5 μg/fly, similar to that reported by Razzag et al. (22).

**ATPase Activities of IFM and Emb<sub>18</sub> Myosins—Drosophila**

IFM myosin exhibited a high Ca-ATPase activity, of 7.5–9 s<sup>-1</sup> (Table I). These values are the same as for phosphorylated myosin from whole *Drosophila* thoraces (36). The Ca-ATPase assay conditions, selected to maximize ATPase rate, were similar to those used by Takahashi et al. (36), except that we used 10 mM calcium rather than 3 mM. The Emb<sub>18</sub> Ca-ATPase activity is similar to that reported for whole larval myosin (26), which was 25% that of their adult myosin mixture (whole thorax). The K<sub>M</sub> for ATP of the Emb<sub>18</sub> myosin Ca-ATPase activity was approximately one-fifth that of the IFM myosin (Fig. 3).

Mg-ATPase activity of the IFM isoform was only 1–2% of the Ca-ATPase activity. The Ca-ATPase and Mg-ATPase activities of transgenic Emb<sub>18</sub> myosin were 20–37% of those obtained with IFM myosin (Table I, Fig. 3). *Drosophila* myosin K-ATPase (EDTA) activity at 0.5 mM KCl was slightly higher than the Mg-ATPase rates (data not shown), and about 50% of the K-ATPase activity reported by Takahashi and Maruyama (26) for whole thorax myosin. Thus, with smTM bound to the myosin, 6.4 μg/fly, similar to that reported for IFM myosin (36), the values for the Emb<sub>18</sub> myosin Ca-ATPase preparations; ~ S.D. of 43 means). For Emb<sub>18</sub> myosin, we obtained a mean displacement of 4.38 ± 2.27 nm (1011 events involving 8 F-actin filaments and 4 myosin preparations; ~ S.D. of 8 means). These estimates are not significantly different (p = 0.605, Student’s t test), but are rather smaller than previously measured with rabbit HMM (5.5 nm) (37) and S-1 (32).

**Sliding Filament Assay—The in vitro motility assay** required a few modifications from the standard rabbit heavy meromyosin (HMM) assay (23) to obtain smooth and continuous movement of actin on *Drosophila* myosin. Actin filaments diffused off the myosin-coated surface after ATP addition unless 0.4% methylcellulose was added to the AB/BSA/glucose/glucose oxidase/catalase and ATP solutions. Actin interactions with myosin were enhanced when the overall ionic strength was lowered to about 25 mM (no added KCl). Higher ionic strengths decreased the number of filaments moving, and some filaments would leave the myosin surface even in the presence of methylcellulose. The best actin movement (greatest number of filaments moving smoothly and continuously) occurred at ~0.5 mg·m<sup>-1</sup>·s<sup>-1</sup> of myosin, which is at the high end of values reported for other myosin types (0.1–0.5 mg·m<sup>-1</sup>·s<sup>-1</sup>; Ref. 28). Movement was not achieved below 0.3 mg·m<sup>-1</sup>·s<sup>-1</sup>. Mean actin velocity on IFM myosin, 6.4 μm·s<sup>-1</sup>, was faster than that typically found with rabbit myosin (4–5 μm·s<sup>-1</sup>; Refs. 23 and 28). Mean actin velocity on IFM myosin was 9-fold faster than with Emb<sub>18</sub> myosin (Table II). Although it was much slower, the embryonic myosin still required methylcellulose to prevent actin diffusion away from the myosin-coated surface, and moved actin best at a myosin concentration of 0.5 mg·m<sup>-1</sup>·s<sup>-1</sup>.

Surprisingly, the binding of smTM to actin increased filament velocity over Emb<sub>18</sub> myosin almost 6-fold (Table II, Fig. 4A). In contrast, smTM had a slight inhibitory effect on IFM myosin driven movement, reducing the velocity (Fig. 4B) and decreasing the number of filaments that moved smoothly and uniformly. Reduced movement quality was not observed with Emb<sub>18</sub> myosin. Thus, with smTM bound to the *Drosophila* IFM actin, the difference in sliding velocity between IFM and Emb<sub>18</sub> myosins was only 1.4-fold.

**Myosin Displacement Size**—The displacements produced by the two *Drosophila* myosins in single molecule interactions with an F-actin in the optical trap are shown in Fig. 5. A short section of signal recording the position of a bead (one of a bead pair attached to F-actin) is shown (Fig. 5A), which is similar to those presented previously for other myosins (32, 37). There are periods of reduced noise due to the increased stiffness when myosin, on the third bead, binds to the actin filament. Signal noise is due to thermal fluctuation of the bead held in the optical trap. The amplitude of the thermal fluctuations was estimated from a running variance (lower trace), which is used to objectively and automatically identify individual myosin attachment events. A histogram (Fig. 5B, gray) shows the distribution of bead positions during periods when no myosin is attached. This noise can be fitted with a Gaussian distribution whose S.D. is defined by the combined stiffness of the optical tweezers (σ = √(kT/k<sub>trap</sub>)). A distribution of individual mean displacements produced by *Drosophila* IFM myosin attachments is shown (Fig. 5B, black). These data are well fit by a Gaussian distribution of the thermal noise of the unattached bead-actin-bead assembly, but stiffened in the x axis by the size of the myosin working stroke. These are representative data from all the interactions of an individual F-actin bead pair. The shift in position of this distribution from the zero position on the x axis estimates the average size of the myosin displacements (37). For the *Drosophila* IFM myosin this produces a displacement of 3.91 ± 2.36 nm (mean of 3547 attachment events obtained from 43 F-actin filaments on a total of 13 IFM myosin preparations; ± S.D. of 43 means). For Emb<sub>18</sub> myosin we obtained a mean displacement of 4.38 ± 2.27 nm (1011 events involving 8 F-actin filaments and 4 myosin preparations; ± S.D. of 8 means). These estimates are not significantly different (p = 0.605, Student’s t test), but are rather smaller than previously measured with rabbit HMM (5.5 nm) (37) and S-1 (32).

Fig. 5C shows the distribution of displacement means (binned into 2-nm bins) for each actin filament interacting with either Emb<sub>18</sub> (gray) or IFM (black). We found that data collected from different pedestals (third bead) with the same F-actin filament had a large variation in mean displacement. Because of this large variability between different estimates, we performed additional control experiments. 1) Only *Drosophila* myosin preparations that demonstrated good quality movement in the in vitro motility assay were used for step size measurements. 2) Step size of rabbit full-length myosin was also measured under the same conditions (Fig. 5C). This gave a step size of 4.20 ± 0.93 nm (mean ± S.D. of 8 filament means, each with >25 events), which is not significantly different from the values for the *Drosophila* isoforms (p = 0.832, one-way analysis of variance). 3) Neither using rabbit actin instead of IFM actin nor adding tropomyosin affected the step size of either *Drosophila* myosin.

The duration of myosin attachments to F-actin in the optical tweezers traces provides a measure of the second order rate constant of A.M + ATP → A + M·ATP. Since the rate of detachment is so fast once ATP binds, the rate constant esti-
Indirect flight muscle myosin from or myosin mutants (6, 38) or by higher ionic strengths (39, 40). or when actomyosin affinity is reduced, either by various actin...probability of further interactions with myosin heads. It thus...Methylcellulose enhances solution viscosity, reducing diffusion much lower actin affinities than rabbit skeletal muscle myosin.

**MATERIALS AND METHODS**

We have demonstrated that myosin can be isolated and purified from *Drosophila* IFMs in sufficient quantities for optical trapping, *in vitro* motility, and ATPase assays. More significantly, we can isolate other myosin isoforms by expressing them transgenically in the IFMs of mutant flies, Mhc10, which do not express the native IFM myosin. Myosin isoform differences do not appear to influence assembly properties (17, 18). Even though embryonic isoforms cause myofibrillar breakdown and a reduction in myosin content as the flies age (17, 18), yields of *Emb*18 from young flies (1–2 days old) were no less than 50% compared with wild type.

**Actin Velocity in Vitro and in Vivo**—Both *Drosophila* myosins move actin *in vitro* only at high surface myosin concentrations, in the presence of methy cellullose and at low ionic strength. All these factors suggest that these myosins have much lower actin affinities than rabbit skeletal muscle myosin. Methylcellulose enhances solution viscosity, reducing diffusion of actin filaments away from the surface and increasing the probability of further interactions with myosin heads. It thus maintains movement at very low myosin surface densities (27) or when actomyosin affinity is reduced, either by various actin or myosin mutants (6, 38) or by higher ionic strengths (39, 40). Indirect flight muscle myosin from *Lethocerus* (waterbug) has a $K_m$ for actin, measured from actin-activated ATPase studies, which is 6–15 times greater than vertebrate fast muscle (41). Thus, it seems likely that IFM myosins may generally have low actin affinities. Since *Emb*18 has similar *in vitro* motility requirements, the low actin affinity likely extends also to the embryonic isoform, normally expressed in the supercontractile muscles of the larva.

Our results show that *Drosophila* IFM myosin produces one of the fastest *in vitro* actin sliding velocities reported for a skeletal muscle isoform. It is faster than rabbit skeletal myosin (4–5 μm s⁻¹) under similar conditions and temperature (27, 42). The high *in vitro* velocities produced by the IFM myosin are consistent with its role in powering one of the fastest contracting *Drosophila* skeletal muscle types. *Drosophila* IFMs produce oscillatory contractions of ~200 Hz at 22 °C (43) and shorten by about 3.4% (44). The thick and thin filaments of each set of IFMs, the opposing dorso-longitudinal and dorso-ventral muscles, slide past each other for up to 2.5 ms during a contraction. Starting with a sarcomere length at rest (45) of 3.4 μm, the length change for each half sarcomere will be 58 nm and contraction velocity will be around ~23 μm s⁻¹, almost 4 times the speed measured in the motility assay. In comparison, rabbit psoas muscle shortens about 7 μm s⁻¹ per half sarcomere at 22 °C (46, 47) or about 3-fold slower than *Drosophila* IFMs.

It is clear from the *Drosophila* IFM myosin data, as previously observed with rabbit skeletal myosin, that *in vitro* velocity tends to underestimate the values of $V_{max}$ achieved *in vivo* (47). This is partly due to the random orientation of cross-bridge heads (48) and because the filaments are not fully unloaded (e.g. binding of other myosin heads) *in vitro*. Further, *in vitro* velocities are usually higher with a full complement of thin filament accessory proteins (28).

The mean velocity produced by the *Emb*18 myosin is 9-fold slower than the IFM isoform and correlates with the slower contraction velocities of the larval body wall muscles. *Emb* myosin is normally expressed in the supercontractile dorsal oblique muscles that have long sarcomeres and a high degree of shortening (49). The mechanical properties of these muscles are unknown, but they are used to power crawling at 1 mm s⁻¹ with individual contractions visible to the naked eye, and must have much slower contraction velocities than IFMs. The actin velocity on *Emb*18 myosin that we have measured more closely resembles those described for phasic smooth muscle isoforms than those of vertebrate slow skeletal muscle isoforms (4, 7, 8).

Although the functional measures of *Emb*18 correlate with the mechanical properties of its native muscles, it should be noted that due to transgenic expression the *Emb*18 isoform has the IFM-specific essential light chain. The *Drosophila* genome contains single copies of both the regulatory and essential muscle myosin light chain genes. The IFMs express a unique essential light chain isoform that differs in 14 amino acids of the C terminus due to alternative splicing of its transcript (50). As light chain isoforms influence velocity (51), the properties of the endogenous embryonic isoform may differ slightly from those measured for *Emb*18. However, since the IFM and the...
transgenically expressed Emb\textsuperscript{18} contain the same essential light chain, any functional differences we observed must derive from the myosin heavy chain sequence.

The binding of smooth muscle tropomyosin dramatically increases actin velocity on Emb\textsuperscript{18} myosin but slightly decreased the sliding rate on IFM myosin. The molecular basis for this potentiation, which has also been found for actin activation of myosin ATPase (52, 53) remains generally unexplained. The effect varies according to myosin type and isoform. Smooth muscle tropomyosin increases actin sliding velocity \(-10\)-fold with phosphorylated Limulus striated muscle myosin (54) and \(2–4\)-fold on smooth muscle myosin (55), but only slightly increases velocities on rabbit skeletal striated muscle myosin (56). On this basis the Emb\textsuperscript{18} isoform acts like a smooth muscle myosin, while the IFM myosin behaves more like rabbit skeletal myosin.

**IFM Myosin ATPase Rates**—The fast nature of the IFM myosin isoform was supported by ATPase measurements. The Ca-ATPase rate of the IFM isoform was \(2–4\)-fold higher than rabbit skeletal muscle and Lethocerus flight muscle myosins measured under similar conditions (36, 57). The significance of the \(K_m\) difference for ATP of the Ca-ATPase activation for the two isoforms is not immediately apparent, since ATP concentrations \textit{in vivo} are vastly in excess of \(1 \mu M\). However, it will be interesting to determine which alternative exon influences this property, even if it does not impact the fly physiologically.

White \textit{et al.} (41) measured Mg-ATPase values of \(0.01–0.05\) s\(^{-1}\) for Lethocerus myosin subfragment 1, S-1, at 20 °C, pH 7; this is about the same as our \textit{Drosophila} IFM myosin value (0.1 s\(^{-1}\)). \textit{Drosophila} IFM ATPase activity correlates with IFMs being classified as fast muscles. However, Lethocerus flight muscles, which are physiologically similar, only contract at 30 Hz, yet have nearly the same myosin ATPase activities. Clearly, myosin ATPase activity does not scale with contraction velocity within this muscle type. This observation is very similar to the results of Molloy \textit{et al.} (58), who showed that IFM fiber ATPase activity is constant across a wide range of insect wing beat frequencies, with the exception of the wasp.

**Single Molecule Mechanics**—There were no significant differences in the overall mean displacements measured for rabbit, IFM, and Emb\textsuperscript{18} full-length myosins. However, there was large scatter in the individual estimates of mean step size obtained for each myosin isoform. Separate estimates of the mean step size were obtained from each third bead tested (and therefore each individual myosin molecule). The individual binding events used to obtain each estimate had an amplitude distribution that was Gaussian with S.D. similar to that of the free bead movement (as described by Molloy \textit{et al.} (31)). The new feature in the data that we report here is that the mean estimates (some obtained using the same actin filament but testing a different myosin molecule of the same isoform) have a greater standard error of the mean than expected on the basis of sampling statistics. In other words, trials on the same myosin type gave results that were significantly variable between myosin molecules tested. This discovery implies that the individual myosin molecules being tested behave differently in this assay. There are three possible interpretations. 1) The myosin molecules are biochemically heterogeneous as a result of differential post-translational modifications; 2) the \textit{Drosophila} myosins occupy a wider range of functionally active molecular orientations in the way they are deposited on the third bead substrate, some of which restrict the production of the full displacement; or 3) the myosin step size depends upon the geometry of myosin binding to actin and the degrees of freedom of movement within the myosin molecule. Any single myosin molecule would give a movement proportional to the cosine of the angle that it makes to the actin filament (as suggested by Molloy \textit{et al.} (Ref. 31) and as shown by Tanaka \textit{et al.} (Ref. 48)). Since in these assays the myosin head orientation is random with respect to the F-actin axis, different flexibilities within the myosins might produce step size distributions with different mean variance. On the basis of our data set, it is not possible to
discriminate among these models. However, it does appear that, in these experiments, the insect myosin step sizes give a broader distribution than rabbit myosin.

The Functional Role of MHC Alternative Exons—At least one of the five MHC regions encoded by alternative exons must be responsible for the observed differences in actomyosin kinetics between the IFM and Emb\(^{18}\) isoforms. The remainder of the molecule is identical between these two isoforms as it arises from constitutive exons of the same gene, and because we engineered the adult tailpiece onto Emb\(^{18}\). Based on mapping the four motor domain alternative exons (exons 3, 7, 9, and 11) onto the three-dimensional chicken skeletal myosin head (16), we currently believe that exon 7 may be responsible for the measured differences in ATPase rates as it encodes part of the lip of the nucleotide site and is part of “switch one.” Exons 11 and/or 3 could contribute to the differences in actin sliding velocity, as they are located near the pivot point of the lever arm (Fig. 1) and have been proposed previously to affect step size (17).

None of the four alternative exon regions in the myosin head corresponds to the loop 1 and loop 2 regions that have been investigated as possible sources of functional variation between myosins from different muscle types (4–9). The seven-aminoc acid insert, which determines differences in ATPase rate and \(v_{\text{in vitro}}\) motility velocity between the tonic and phasic smooth muscle isoforms, is not within a homologous region encoded by a \textit{Drosophila} alternative exon (7, 8). Thus, multiple mechanisms appear to have evolved to modify myosin function.

In summary, we have observed dramatic differences in the \(v_{\text{in vitro}}\) motilities, the effects of smooth muscle tropomyosin on motility, Ca- and Mg-ATPases, and \(K_m^\text{ATP}\) for ATP between the IFM and Emb\(^{18}\) myosin isoforms. These differences are certainly sufficient to explain why the Emb\(^{18}\) isoform cannot functionally substitute for the native isoforms in the flight muscle. Most obviously, the Emb isoform is not fast enough to power flight. Although the details of how these functional differences arise (e.g. different ADP release rates) remain to be investigated, we have shown that functional myosins can be expressed and purified from the IFMs in sufficient quantities for biochemical experiments. This will enable us to develop more sophisticated assays to determine which steps in the cross-bridge cycle are different between these two isoforms.

By exchanging alternative exons between the IFM and Emb\(^{18}\) isoforms, we can relate the functional differences described here to specific structural regions of the myosin heavy chain. These alternative regions can then be examined in more detail using site-directed mutagenesis to determine specific amino acids or groups of residues that are responsible for particular functional modifications. The advantage of the \textit{Drosophila} \textit{Mhc} system for this analysis of myosin is that we can, in transgenic organisms, observe the effects from the level of myosin molecular properties, through muscle mechanics (20, 33) to whole animal locomotory performance (17) to gain a fully integrative understanding of the role of the alternative exon regions.

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