Kinetic Analysis of Drosophila Muscle Myosin Isoforms Suggests a Novel Mode of Mechanochemical Coupling*

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The molecular mechanism of myosin function was addressed by measuring transient kinetic parameters of naturally occurring and chimeric Drosophila muscle myosin isoforms. We assessed the native embryonic isoform, the native indirect flight muscle isoform, and two chimeric isoforms containing converter domains exchanged between the indirect flight muscle and embryonic isoforms. Myosin was purified from the indirect flight muscles of transgenic flies, and S1 was produced by a-chymotryptic digestion. Previous studies in vertebrate and scallop myosins have shown a correlation between actin filament velocity in motility assays and cross-bridge detachment rate, specifically the rate of ADP release. In contrast, our study showed no correlation between the detachment rate and actin filament velocity in Drosophila myosin isoforms and further that the converter domain does not significantly influence the biochemical kinetics governing the detachment of myosin from actin. We suggest that evolutionary pressure on a single muscle myosin gene may maintain a fast detachment rate in all isoforms. As a result, the attachment rate and completion of the power stroke or the equilibrium between actin-myosin-ADP states may define actin filament velocity for these myosin isoforms.

Cyclical shortening and lengthening of a muscle fiber is the direct result of myosin cross-bridges transiently interacting with actin filaments utilizing the energy from ATP hydrolysis. Specific muscle fibers show characteristic biochemical and mechanical properties that are largely governed by the isoform of myosin expressed (1, 2). It is widely accepted that the overall cross-bridge cycle of muscle myosin is conserved; thus alterations in this cycle are the result of changes in the biochemical kinetic constants of individual events resulting directly from myosin sequence changes. Recent work supports the inference that in Drosophila muscle myosin isoform functional diversity is the result of kinetic differences governing strong binding attachment and/or detachment kinetics rather than changes in step size (3–5). Structural studies have focused on identifying regions of the myosin molecule, in both muscle and non-muscle isoforms, containing naturally occurring variations that could account for these isoform-specific properties (6–11). Functional studies of these natural variants along with intentionally modified myosin isoforms have begun to probe the structure-function relationship critical to elucidating the molecular mechanism of myosin (for reviews, see Refs. 12 and 13).

To study specific regions and amino acids within myosin that define the complex structure-function relationship, we have turned to the genetically tractable organism, Drosophila melanogaster. This model system allows the creation of transgenic organisms containing altered regions or amino acids within myosin to probe the functional contribution of these residues in the mechanochemical cycle (14). Recent studies have shown that Drosophila muscle-specific myosin isoform properties are determined by alternative exon usage (4). These myosin isoform differences modulate the mechanical performance of the muscle fiber in which they are expressed (15). A kinetic analysis of the Drosophila native and mutant myosin isoforms will yield further insight into the molecular mechanism underlying myosin function.

To date, at least 15 different muscle myosin heavy chain isoforms are known to be expressed in Drosophila (16–19), each produced from alternative splicing of transcripts generated from a single gene. Isoform differences are coded for by six sets of alternative exons (16) that generate amino acid sequence variation; four of these regions are found in the catalytic domain, one is found in the hinge region of the rod region, and one is found at the C terminus of the rod region (for an overview, see Ref. 9). We chose to study two native myosin isoforms that possess differences in all six regions of sequence variation (4): an embryonic body wall muscle (EMB) isoform, expressed in muscle important for larval locomotion and presumably a slow twitch fiber type (20), and the indirect flight muscle myosin (IFI) isoform, expressed in the fast type oscillatory muscle that powers flight (21, 22). In addition, we studied two chimeric myosin isoforms in which the converter domain was genetically swapped between the IFI and EMB isoforms (Fig. 1) to create IFI-EC (IFI isoform with embryonic converter

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The abbreviations used are: EMB, embryonic myosin; IFI, indirect flight muscle myosin; IFI-EC, IFI with embryonic converter domain; EMF-IC, EMB with indirect flight muscle myosin converter domain; IFM, indirect flight muscle; cm-ADP, coumarin-ADP (3′-O-β-N-[2-(7-diethylaminomethylamino)-carboxamidoethyl]carbamoyl)ADP); cATP, caged ATP (adenosine 5′-triophosphate(1-2-nitrophenyl)ethyl ester); DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid; Ap,A, P1-P8-di(adenosine 5′)-pentaphosphate; S1, subfragment 1.

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**MATERIALS AND METHODS**

**Drosophila Myosin Isolation and S1 Production**—Myosin was isolated from the IFM of 150–180 wild-type or transgenic flies (those expressing the ENM, EMB-IC, or IFI isoforms) as described previously (4). The final myosin pellets were resuspended in 75–100 μl of digestion buffer (120 mM NaCl, 20 mM Na₂PO₄, pH 7.0, 1 mM EDTA, and 4 mM DTT), and the production of S1 by α-chymotrypsin digestion was carried out using a method based on Silva et al., (29). Resuspended myosin was incubated at 20 °C for 5 min and then incubated with 0.2 mg/ml α-chymotrypsin for 6 min. To quench the reaction, phenylmethylsulfonyl fluoride was added to a final concentration of 1.5 mM. Subsequently, the reaction was centrifuged at 68,000 rpm (TLA 100.3 rotor) for 25 min in a Beckman ultracentrifuge to pellet the undigested myosin and myosin rods. The supernatant containing the S1 was removed and diluted to 500 μl with low salt experimental buffer (30 mM KCl, 5 mM MgCl₂, 20 mM MOPS, pH 7.0, and 4 mM DTT). To concentrate the S1, the samples were centrifuged at 12,000 × g with a Sorvall MC 12V at 4 °C using a Millipore Ultrafree 0.5 μm centrifugal filter with a 5,000 kDa cut-off. The final volume of the supernatant was 30–50 μl containing a S1 concentration of 20–30 μM. The S1 samples were stored on ice and used within 2 weeks. S1 conversion was determined from the absorbance at 280 nm (ε²⁻⁸ = 0.75 cm⁻¹) and the molecular mass of 115 kDa.

**Actin**—Rabbit skeletal actin was prepared according to the method of Spudich and Watt (30). The concentration was determined based on a molecular mass of 42 kDa and the absorbance at 280 nm (ε²⁻⁸ = 1.104 cm⁻¹, Ref. 49). The actin filaments (10 μM) were stabilized by mixing them with equimolar concentrations of phallolin and incubating for at least 4 h prior to use.

**Chemicals**—Caged ATP (cATP) was purchased from Molecular Probes. cm-ATP was a gift from Martin Webb (National Institute for Medical Research, Mill Hill, London, UK) and prepared according to Webb and Corrie (31).

**Flash Photolysis Set-up**—The flash photolysis system used in this work was described previously (32). Briefly, the 20-μl sample is held in a quartz cuvette, and ATP is released from cATP by a single 5-ns flash from a neodymium-yttrium-aluminum-garnet laser (Surelite I-10, 70 mJ maximum power) at 353 nm along the vertical axis of the cell. This liberates up to 50 μM ATP from 0.5 mM cATP at a rate of 90 s⁻¹. The cuvette allows simultaneous monitoring of the absorbance and 90° light scattering (or fluorescence) changes following the laser flash. The sample was exposed to white light >389 nm (from a 100-watt halogen lamp). The absorbance was measured at 405 nm via a monochromator, and the rate and quantity of ATP liberated in each laser flash was determined. Light scattering was monitored using the white light and records the dissociation of the actomyosin or acto-S1 complex on release of ATP. Apart from the components of the low salt experimental buffer (described above), the 20-μl samples contained 10 mM DTT, various ATP concentrations, and either 2 units/ml apyrase (ATP-induced dissociation of acto-S1) or a glucose-hexokinase system (0.03 units/ml hexokinase, 1 mM glucose, and 100 μM α-MG₆₅) with a total ATPase activity measured. Measurements were repeated in the presence of 500 nM NAD⁺ and then centrifuged at 300,000 × g at 4 °C for 30 min at 20 °C to determine K₄₅. Measurements were repeated in the presence of 10 mM ATP with 500 nM S1 and actin concentrations between 0 and 5 μM to determine K₄₅. The supernatant was separated from the pellet at each actin concentration, and the pellet was re-equilibrated in low salt solution of water. All samples were applied to a 10% SDS gels. Gels in the absence of nucleotide (100 nM S1) were silver-stained (Bio-Rad) to visualize the protein bands, while those obtained in the presence of 10 mM ATP (500 nM S1) were stained with Coomassie Blue. The density of each protein

**FIG. 1. Structure of myosin S1 and Drosophila converter domain primary amino acid sequence.** a, sequences for isovariants 11E (IFI isoform) and 11C (EMB isoform) with nonconserved amino acids marked with an asterisk (*). b, crystal structure of the chicken skeletal muscle myosin S1 fragment minus the light chains. The portion of the converter domain (chicken skeletal residues 712–779) encoded by Drosophila exon 11 (residues 724–784) is shown in blue. Residues 731–738 are unresolved in this crystal structure.

Drosophila Myosin Isolation and S1 Production—MYOSIN was isolated from the IFM of 150–180 wild-type or transgenic flies (those expressing the EM1, EMB-IC, or IFI-EC myosin isoforms in the IFM) as described previously (4). The final myosin pellets were resuspended in 75–100 μl of digestion buffer (120 mM NaCl, 5 mM Na₂PO₄, pH 7.0, 1 mM EDTA, and 4 mM DT1), and the production of S1 by α-chymotrypsin...
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SCHEME 1. The interaction of S1 with actin, ATP, and ADP. M, A, T, and D symbolize the myosin S1, actin, ATP, and ADP, respectively. $K_a$ is the association constant for ATP binding in step 1, $k_{-2}$ is the rate constant of the protein isomerization limiting actin dissociation from myosin-ATP, and $K_{AD} = (k_{-AD}/k_{AD})$ is the equilibrium constant for ADP binding.

band was quantified and used to determine the affinity of S1 for actin. The actin-bound S1 concentration was estimated from the pellet S1 band densities or from the supernatant S1 band densities by subtracting them from the S1 density measured in the absence of actin where all the S1 remained in the supernatant. The [bound S1] versus [actin] plots were analyzed with the following equation (34),

$$[A]_0^F - ([A]_0 + [S1]_0 + K_F + [S1]_0) = 0$$

where $[S1]_0$ and $[A]_0$ are the total S1 and actin concentrations, respectively, and $F$ is the fraction of bound S1 (acto-S1/[S1]$_0$). Analysis of the Kinetic Data—The kinetic scheme for the interaction of actin and S1 with ATP and ADP is described in Scheme 1. The following equation was derived from the scheme and used to determine the value of $K_{AD}$,

$$k_{obs} = k_c [ATP]/(1 + [ADP]/K_{AD})$$

where $k_{obs}$ represents the observed rate constant for the ATP-induced acto-S1 dissociation reaction, $K_c$ is the second order rate constant for the binding of ATP to acto-S1, and $K_{AD}$ is the equilibrium dissociation constant for ADP binding to acto-S1. The relative rate constants ($k_{obs}$) for Fig. 7 were calculated as $k_{cat} = k_{obs}/k_e$ where $k_e$ is the value measured in the absence of ADP.

**RESULTS**

Production of Drosophila S1—EMB and chimeric converter myosin isoforms were isolated from the IFMs of transgenic flies expressing a single isoform in an IFM null mutant (35). The procedure of Silva et al. (29) was modified to produce a more concentrated and pure solution of S1 (Fig. 2). The previous study of Silva et al. (29) used dialyzed S1 samples containing measurable quantities of actin, myosin rods, and other proteolytic fragments. The 10% SDS gel showed no detectable contamination of the S1 samples with actin or other proteins. In addition, the clarified S1 was concentrated rather than dialyzed to exchange the buffer, eliminating possible loss of the S1 fragment. The regulatory light chain is lost (Fig. 2) following α-chymotrypsin digestion, but the essential light chain is intact. Drosophila myosin S1 isoforms were produced in sufficient quantities (60–150 μg) to perform experiments utilizing the flash photolysis/light scattering method (32).

**$Mg^{2+}$ ATPase Activities**—Basal Mg$^{2+}$ ATPase activity of the IFI isoform was 4-fold faster (0.083 ± 0.017 s$^{-1}$) than that for the EMB isoform (0.022 ± 0.004 s$^{-1}$) (Table I), similar to the 5-fold difference found previously for basal Mg$^{2+}$ ATPase activities of the full-length native Drosophila myosin isoforms (Table I) (4). The chimeric converter S1 isoforms showed little difference in the basal Mg$^{2+}$ ATPase activities when compared with the parent backbone. IFI-EC S1 activity was 0.098 ± 0.028 s$^{-1}$, similar to IFI isoform activity, while EMB-IC activity was 0.014 ± 0.003 s$^{-1}$, slightly lower than EMB isoform activity. These Mg$^{2+}$ ATPase activities were in good agreement with the trend reported for full-length myosin isoforms (IFI-EC > IFI > EMB > EMB-IC) (5).

The actin-activated Mg$^{2+}$ ATPase activities were determined in the presence of increasing actin concentrations, and all four isoforms showed linear increases in activity at the measured actin concentrations. The initial slope of Mg$^{2+}$ ATPase activity versus [actin] plots gives an estimate of the ratio $k_{cat}/K_m$ where $k_{cat}$ is the maximum actin-activated Mg$^{2+}$ ATPase activity and $K_m$ is the actin concentration required for half-maximal activation (Table I). The actin activation of Mg$^{2+}$ ATPase activity was greater for IFI S1 than for EMB S1, and the extent of actin-activated Mg$^{2+}$ ATPase activity of the converter chimeric isoforms is identical to that observed for the parent S1.

The Affinity of S1 for Actin—The affinity of the S1 isoforms for actin was measured using the co-sedimentation method. In the absence of nucleotide, incubation of 100 nM IFI S1 with increasing actin concentrations (Fig. 3b) indicates a tight affinity (<10 μM) of S1 for actin ($K_a$). The plot was generated after quantitation of the S1 and actin bands on SDS gels of both the supernatants and pellets (e.g. Fig. 3a). Characterization of the rigor actin affinity of the other three isoforms suggests similar tight affinities. Quantitative affinities ($K_a$) could not be determined for any isoform using this method given the tight binding, represented in Fig. 3b as a dotted line. In the presence of 10 μM ADP, the affinity of S1 for actin ($K_a$) was weakened for all isoforms (Table I); a representative plot of IFI S1 density versus [actin] in the presence of ADP is shown (Fig. 3c).

**cm-ADP dissociation from S1**—To estimate the rate constant for ADP dissociation from IFI S1 in the absence of actin we used a fluorescent analogue of ADP and a novel flash photolysis method described recently (36). IFI S1 (4 μM) was incubated with 10 μM cm-ADP and 100 μM cATP, and the S1-bound cm-ADP was displaced by releasing ATP (15 μM) from cATP (100 μM) with a single laser flash. The coumarin fluorescence was monitored, and the transients were fitted with a single exponential function (Fig. 4) and gave a $k_{obs}$ value of 7.5 ± 1.3 s$^{-1}$. The $k_{obs}$ was ATP concentration-independent between 15 and 75 μM indicating that ATP effectively displaced cm-ADP, and the $k_{obs}$ values corresponded to the dissociation rate constant ($k_{-p}$) of cm-ADP from S1. Similar cm-ADP displacement experiments were repeated with IFI-EC, EMB-IC, and EMB S1 samples and gave $k_{-p}$ values of 8.4 ± 1.7, 1.7 ± 0.3, and 1.8 ± 0.4 μM$^{-1}$ s$^{-1}$ for the IFI-EC, EMB-IC, and EMB S1, respectively (Fig. 4 and Table II). The IFI cm-ADP dissociation rate constant was 4-fold faster than from EMB S1, and swapping the converter domain yielded no significant difference in the cm-ADP release rate.

We observed a decrease in the amplitude of the transient as cATP concentration increased indicating that cATP could compete with cm-ADP for binding to IFI S1. The cm-ADP concentration was 10 μM in the experiments, and a plot of amplitude...
Table I

Biochemical and mechanical measurements for S1 isoforms

| Myosin isoform | IFI | IFI-EC | EMB | EMB-IC |
|----------------|-----|--------|-----|--------|
| Basal Mg\(^2\+\) ATPase rate (head\(^{-1}\) s\(^{-1}\), S1 | 0.083 ± 0.017 | 0.098 ± 0.028 | 0.022 ± 0.004 | 0.014 ± 0.003 |
| Basal Mg\(^2\+\) ATPase rate (head\(^{-1}\) s\(^{-1}\), whole myosin | 0.11 ± 0.01 | 0.4 ± 0.04 | 0.222 ± 0.002 | 0.06 ± 0.03 |
| \(k_{obs}/K_a (10^9 M^{-1} s^{-1})\) | 8.5 ± 3.4 | 8.6 ± 2.6 | 3.4 ± 1.4 | 2.3 ± 0.9 |
| \(f_{max} (Hz)\) | 109 ± 5 | 47 ± 1 | 17 ± 1 | 35 ± 1 |
| \(r_s (s^{-1})\) | 1059 ± 63 | 729 ± 30 | 90 ± 5 | 367 ± 26 |
| Actin velocity (\(V_{actin}\) (μm/s)) | 6.4 ± 0.7 | 2.7 ± 0.1 | 0.7 ± 0.1 | 5.4 ± 0.2 |

\(^a\) From Swank et al. (4, 15).

\(^b\) From Littlefield et al. (5).

The affinity of S1 for actin was characterized with the co-sedimentation method (see "Materials and Methods"). A representative SDS-polyacrylamide gel of the pellets from experiments where IFI S1 (100 nM) was mixed with actin (0, 0.03, 0.06, 0.1, 0.25, 0.5, 1.5, and 9 μM) in the absence of nucleotide. The densities of the IFI S1 bands as a function of [actin] in the absence (○) and presence of ADP (10 μM) (□) are also shown. In b, the dashed line indicates what would be expected for very tight S1 binding to actin; this tendency is exhibited by the data. arb., arbitrary.

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The affinity of actin affinity in the presence (\(K_{apo}\)) and absence (\(K_A\)) of ADP (Scheme 2). The affinity of S1 for actin was characterized with the co-sedimentation method (see “Materials and Methods”). A, representative SDS-polyacrylamide gel of the pellets from experiments where IFI S1 (100 nM) was mixed with actin (0, 0.03, 0.06, 0.1, 0.25, 0.5, 1.5, and 9 μM) in the absence of nucleotide. The densities of the IFI S1 bands as a function of [actin] in the absence (○) and presence of ADP (10 μM) (□) are also shown. In b, the dashed line indicates what would be expected for very tight S1 binding to actin; this tendency is exhibited by the data. arb., arbitrary.

versus [cATP] could be described by a hyperbolic fit with \(K_{apo}\) at 100 μM cATP (Fig. 4a, inset). Accordingly, in the presence of 10 μM cm-ADP and 100 μM cATP, the S1 bound equal amounts of cm-ADP and cATP, which indicates that the affinity of cATP for IFI S1 was 10 times weaker than that of cm-ADP. Assuming a \(K_a\) value for cm-ADP of 7.5 μM (see “Discussion”) this suggests cATP affinity of 75 μM. Both values are much weaker than the value for mammalian skeletal muscle S1 (\(K_p^{cATP} = 10 μM\) and \(K_p^{cMg-ADP} = 0.3 μM\), Ref. 36).

ATP-induced Acto-S1 Dissociation—To determine the second order rate constant (\(K_r^{+2}\), refer to Scheme 1) for the ATP-induced dissociation of the acto-S1 complex, we mixed S1 (1–3 μM) with actin (1 μM), and the acto-S1 complexes were dissociated by releasing ATP from cATP (initially 500 μM) with a series of decreasing intensity laser flashes on a single sample. The changes in light scattering were recorded, and the transients were fitted with single exponential functions (Fig. 5). For each S1 isoform a \(k_{obs}\) versus [ATP] plot was created, and the slope of a linear fit gave a value for \(K_r^{+2}\) (Fig. 6). These second order rate constants were \(0.75 ± 0.08, 0.84 ± 0.09, 0.51 ± 0.05, and 0.91 ± 0.13 \times 10^6 M^{-1} s^{-1}\) for IFI, IFI-EC, EMB-IC, and EMB, respectively. The value measured for the IFI S1 isoform was similar to that published previously (0.82 ± 10^6 M^{-1} s^{-1}, Ref. 29).

The Affinity of Acto-S1 for ADP—Competition between ADP and ATP for binding to acto-S1 provides a method for determining the dissociation equilibrium constant for ADP binding to acto-S1 (\(K_{AD}\), refer to Scheme 1). To determine \(K_{AD}\), the ATP-induced acto-S1 experiments at fixed ATP concentration were repeated in the presence of different concentrations of ADP, which inhibits this dissociation as the concentration increases (for details refer to Ref. 32). S1 (1–3 μM) was mixed with actin (1 μM) and ADP (0–1.5 mM), and 40 μM ATP was released from cATP (500 μM) with each laser flash. The recorded light scattering transients were fitted with single exponential functions (data not shown) to determine the \(k_{obs}\) values. Hyperbolic fits (Equation 2) to the \(k_{obs}\) versus [ADP] plots (Fig. 7) gave \(K_{AD}\) values of 409 ± 26, 587 ± 48, 418 ± 18, and 838 ± 75 μM for IFI S1, EMB S1, EMB-IC S1, and IFI-EC S1, respectively (Table II). The \(K_{AD}\) value for IFI S1 (409 ± 26 μM) was similar to that previously published (398 ± 28 μM, Ref. 29).

Discussion

Properties of Native and Chimeric S1 Isoforms—The work presented here demonstrates that it is possible to isolate high quality myosin subfragment 1 from transgenic Drosophila in sufficient quantities for a transient kinetic analysis. The quality of the preparation is demonstrated by SDS-PAGE, basal Mg\(^2\+) ATPase data, and actin binding titrations. The SDS-polyacrylamide gel (Fig. 2) indicates no contaminant protein, no significant proteolysis side products, and the presence of the intact essential light chain. Successful isolation of protein requires less than 2 working days, minimizing the loss from cellular proteases. Once purified, the protein is stable on ice for at least 14 days. This procedure represents a significant quantitative and qualitative improvement over those reported by White et al. (37) for Lethocerus and the recent Drosophila work of Silva et al. (29).

ATPase Activity—Measured Mg\(^2\+) ATPase activities for a series of preparations over the last year indicate a consistency in the amount of active protein in each sample (see S.D. in Table I). Previous work has shown that IFI S1 Mg\(^2\+) ATPase activity is comparable to full-length IFI and rabbit fast muscle myosin and S1 under identical conditions (29). We obtained a basal rate for IFI S1 of 0.083 s\(^{-1}\), which is in the same range as that measured for Lethocerus insect flight muscle myosin S1 (0.01–0.05 s\(^{-1}\), Ref. 37). Quantitative differences between basal Mg\(^2\+) ATPase rates of the S1 isoforms and their whole myosin isoforms (Table I) are primarily attributed to differ-
times in salt concentrations and pH in the two assays. The actin-activated Mg\(^{2+}\) ATPase rates for each isoform were linear up to an actin concentration of ~30 μM and represent a 3–5-fold activation over basal rates. Low salt actin-activated ATPase activity for S1 is normally more consistent than that for full-length myosin, which can be affected by the insoluble myosin having restricted access to actin-binding sites in the assay.

**Actin Binding**—The actin sedimentation data demonstrated that rigor actin binding was tight for all isoforms with an affinity of <10 nM. The stoichiometry of binding was 1:1 relative to actin for each isoform, further indicating a high quality preparation (Fig. 3a). The tight affinity at 30 mM KCl is similar to all other myosin motor domains that have been measured (7, 34, 38, 39). The presence of 10 mM ADP, sufficient to saturate the ternary complex, weakened S1 actin affinity and allowed K\(_{DA}\) to be measured. Values for these S1 isoforms, 60–200 nM, were close to those of rabbit S1 under similar conditions (40). The sedimentation data in the presence of ADP show only a single population of myosin heads, which indicates the preparation is substantially free of “dead heads,” i.e. myosin heads that bind tightly to actin and remain so in the presence of nucleotide.

**Nucleotide Binding**—The rate of ADP release from S1 (in the absence of actin) was measured using the fluorescent analog cm-ADP (31, 36) and showed that IFI S1 exhibited a 4-fold greater cm-ADP release rate (k\(_D\)) relative to the EMB isoform (Table II). The value for EMB is similar to the value for rabbit skeletal muscle S1 (36). The quantities of S1 prevented us from estimating the cm-ADP association rate constant, but the 4-fold difference between the k\(_D\) values is likely to correlate with a 4-fold difference between the corresponding ADP affinities for S1 (K\(_{AD}\)) (Table II).

The ADP affinity for acto-IFI S1 (K\(_{AD}\)) was identical to that measured previously by Silva et al. (29), and the ADP release rate from acto-S1 was estimated using the equation
\[
\text{rate} = k_{AD} [\text{S1}] [\text{ADP}] / K_{AD} + [\text{ADP}]
\]

The overall cm-ADP dissociation rate constant was estimated from observed cm-ADP dissociation rates for acto-S1. This value for IFI S1 was identical to that previously published (29), and the ADP release rate for all S1 isoforms is very fast, >4000 s\(^{-1}\) (Table II). The rate of ADP release has been shown to linearly correlate with actin filament velocity in vertebrate myosins (13, 26–28), and we will discuss this issue when considering the mechanical data from the *Drosophila* isoforms.

**Comparison of IFI S1 with EMB and Converter Chimera**

S1—Several parameters measured here varied by less than a factor of 2 between the IFI and EMB isoforms: the ATP-induced dissociation of acto-S1 (K\(_{AD}\)), the affinity of ADP for acto-S1 (K\(_{AD}\)), and the affinity of actin for S1-ADP (K\(_{DA}\)). This indicates that in these two combinations the four alternative exons in the motor domain do not influence these parameters to any major extent. Furthermore the single exon chimeras confirm that two of the five converter domain variants specifically do not have a major influence on these parameters. However, it should be
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Intriguingly the thermodynamic coupling of actin to the parent S1 isoform. The converter domain therefore, perhaps as expected, does not have a major influence on ADP affinity for S1 or the steady-state Mg$^{2+}$ ATPase rate. However, the differential response of $K_{AD}$ and $K_{DP}$ for IFI versus EMB means that the ratio $K_{AD}/K_{DP}$ (termed the thermodynamic coupling between actin and ATP affinities) does show a marked shift. For Drosophila IFI S1 this coupling is $\sim 55$, similar to that measured for rabbit skeletal S1 ($\sim 50$, Ref. 38). This is not unexpected as both isoforms function in fast muscle types. Intriguingly the thermodynamic coupling of Drosophila EMB S1 ($\sim 300$) is 6-fold greater than that of IFI, which is not expected for a slow type myosin. This means that the coupling between actin and nucleotide binding is affected by at least one of the four exon changes between IFI and EMB.

These relatively small effects on the biochemical parameters are in stark contrast with the mechanical parameters measured for the same four isoforms (Table I). In particular, the frequency of maximum work, $f_{max}$ phase 3 of tension recovery after a lengthening step, $r_o$, and the in vitro motility data show an $\sim 10$-fold change between these IFI and EMB parameters. It can always be argued that differences in mechanical data ($r_o$ and $f_{max}$ collected in the isolated muscle fibers of transgenic flies could reflect problems with assembly and mechanical coupling of the myosin to the rest of the sarcomere. However, the in vitro actin velocity data with isolated myosin are independent of such issues. Since all these parameters show the same pattern they are likely to reflect the intrinsic mechanical and biochemical properties of the myosin motor.

For the well studied vertebrate muscle myosins there is a good correlation between the net rate constant for cross-bridge detachment and both the in vitro motility and maximum shortening velocity data (13). This was first pointed out by Siemankowski and colleagues (26) who argued that motility was produced by a large number of myosin molecules interacting with an actin filament, most of which spent only a short time attached to actin (now referred to as the duty ratio, $t_{on}/t_{cycle}$). Hence the net ATPase rate, which is the sum of all steps in the cycle, need not correlate with velocity, which is only governed by the net rate constant for cross-bridge detachment. If attachment and the power stroke are relatively rapid events compared with the cycle time, then the rate of cross-bridge detachment after the power stroke could limit velocity. Siemankowski et al. (26) estimated $k_{min}$, the slowest possible rate constant for an event during the attachment part of the cycle, from $V = k_{min}dS_L$ where $V$ is the fiber velocity (in muscle lengths/s), $d$ is the distance moved by the cross-bridge in a working stroke, and $S_L$ is the half-sarcomere length. This has been restated in recent years in terms of motility data as $V = dl/t_{on}$, where $V$ is

SCHEME 2. The coupling between the nucleotide- and actin-binding sites. Symbols are as in Scheme 1. The affinity of the myosin for actin is described by $K_a$ in the absence of nucleotide and by $K_{AD}$ in the presence of it. The affinity of myosin for ADP is given by $K_d$ and $K_{DA}$ in the absence and presence of actin, respectively. All equilibrium constants are dissociation equilibrium constants defined by the dissociation rate constant divided by the association rate constant ($K_1 = k_{off}/k_{on}$).
velocity in \( \mu \text{m s}^{-1} \), \( d \) is as above, and \( t_{\text{on}} \) is the duty ratio or fraction of the ATPase cycle time the cross-bridge spends attached (\( k_{\text{on}} = 1/t_{\text{on}} \)). Data from a wide range of muscle types have shown a good correlation between \( k_{\text{on}} \) or \( t_{\text{on}} \) and the ADP release rate from acto-S1 (or estimated from ADP affinity) (26–28, 34). We previously argued (29) that \( k_{\text{off}} \), the correct order to define the contraction velocity of the IFM (14.5 muscle lengths/s, \( \text{SL} \)), have shown a good correlation between differences in experimental conditions (42). Whichever velocity measurement is used the EMB isoform is 5–10-fold slower than the IFI, yet \( k_{\text{off}} \) is very similar to that of the IFI. It is clear therefore that \( k_{\text{off}} \) is too fast to limit the velocities measured for EMB. In principle, if ADP release is very fast then detachment could be limited by the following association of ATP and dissociation of actin, controlled by \( K_{b_{\text{ADP}}} \) ATP. For the ATP-induced detachment to be slower than the estimates of \( k_{\text{off}} \), the ATP concentration would need to be <4 mM, which is of the order expected for cellular ATP concentration. But the data presented here also show no correlation between \( K_{b_{\text{ADP}}} \) and velocity for the different isoforms. The conclusion is therefore that the cross-bridge detachment rate does not limit velocity for the EMB isoform.

This conclusion provokes the ancillary question of what then is defining velocity. Within the original analysis of Siemiankowski et al. (26) two possibilities emerge. The first is that the ADP affinity measured here is not appropriate for the cross-bridge cycle in the steady state. Simply adding ADP to acto-S1 could produce an acto-S1-ADP that has weaker ADP affinity (and faster ADP release) than the longer lived complex existing in the steady-state ATPase cycle. The presence of such an ADP complex has been proposed since the pioneering work of Sleep and Hutton (43) and has appeared in several different versions since (38, 39, 44). The central idea is that the strongly bound actin-myosin-ADP complex formed on P1 release must isomerize before ADP can be released, and it is this isomerization that limits velocity. Such additional ADP ternary complexes have been of interest in recent times because of their role in slow, force-holding, or tension-sensing myosins (45–47). A significant role for such a ternary ADP complex in a very fast myosin such as the IFI would be surprising, but it could play a role in a slow myosin such as EMB.

A second possibility, given the function of the IFM is to perform oscillatory work at high frequencies, is that the difference between the IFI and the vertebrate fast myosins reflects this different role. With oscillations of 200 Hz and a short sarcomere length change of 2–5% very few myosin heads may contribute to each wing beat. Therefore, the timing of attachment and completion of the power stroke may be a more important parameter in defining function than the detachment of the cross-bridge, provided this latter parameter remains fast. In support of this hypothesis, a recent study (48) finds that actin velocity deviates from Michaelis-Menten kinetics at high ATP concentrations and suggests that a shift to attachment-limited velocity or a variable ADP release rate can explain this effect. It is also notable that \( r_{\text{p}} \), the rate constant of a mechanical force-generating event linked to \( P_1 \) release, shows better correlation to the velocity data than either \( K_{b_{\text{ADP}}} \) or \( k_{\text{off}} \) (Table 1).

The fundamental point remains that the Drosophila cross-bridge cycle is different in this respect from the other muscles studied to date. It is possible that this reflects the evolutionary constraints imposed on Drosophila for adapting a single myosin gene to different functions rather than divergence of a family of myosin genes. Presumably there is strong evolutionary pressure for fast ADP release rate in the IFI isoform based on the physiological function of the IFM. It is possible that evolutionary pressure produced fast ADP release in the other native isoforms, and a different strategy evolved for functional tuning, which is yet to be elucidated. In addition, we know relatively little about the physiological role of the EMB isoform, making functional conclusions about the alternative exon-induced changes speculative. However, it is clear that the EMB isoform is a much slower myosin but does not appear to be related to a smooth muscle-type myosin having a long lived actomyosin-ADP complex.

**Role of the Converter Domain**—Given the location of the converter domain in the catalytic region of myosin (Fig. 1), it is considered to be central to coupling the energy of hydrolysis with the mechanical events of the power stroke. Swank et al. (15) hypothesized that the actin velocity and fiber mechanical data of the converter chimeras could be most simply explained by changes in kinetics. This was confirmed by Mg\(^{2+}\) ATPase data collected by Littlefield et al. (5), who also found no change in step size. Swank et al. (15) suggested that the converter domain was specifically influencing the detachment rate and most likely the ADP release rate. However, the converter domain exchanges did not have any major influence on any of the biochemical parameters measured here, not unexpected considering the relatively small differences between the native IFI.
and EMB parameters. A key difference between the data presented here and the mechanical studies is that the biochemical kinetics are all measured under zero load. It remains possible that an important role of the converter is in the coupling of mechanical and biochemical events and that these are minimized in the absence of external load. The presence of five isovariants for a subregion of the converter domain in Drosophila (16, 17, 19) suggests that these residues are important for kinetic tuning of expressed myosin isoforms. Since this alternative domain did not significantly influence any of the biochemical parameters measured here, further biochemical studies are necessary to determine the mechanism by which the converter influences actin velocity and fiber mechanics.

In conclusion, the mechanism for functionally tuning the Drosophila myosin isoforms is different from that elucidated for vertebrate myosin isoforms. It will be of great interest to probe other native Drosophila myosin isoforms to verify this lack of correlation between ADP release rate and actin filament velocity.

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