Members of the myosin II class of molecular motors have been referred to as “conventional,” a term used to describe their ability to form thick filaments, their low duty ratio, the ability of individual motor-containing “heads” to operate independently of each other, and their rate-limiting phosphate release. These features ensure that those motors that have completed their power stroke dissociate rapidly enough to prevent them from interfering with those motors that are beginning theirs. However, in this study, we demonstrate that myosin IIB, a cytoplasmic myosin II particularly enriched in the central nervous system and cardiac tissue, has a number of features that it shares instead with “unconventional” myosin isoforms, including myosins V and VI. These include a high duty ratio, rate-limiting ADP release, and high ADP affinity. These features imply that myosin IIB serves a set of physiologic needs different from those served by its more conventional myosin II counterparts, and this work provides a plausible basis for explaining the physiologic role of this unconventionally conventional myosin.

The myosin superfamily currently consists of 17 classes of motors (1). The class II myosins include those motors that compose the thick filaments of smooth, skeletal, and cardiac muscle. Because myosin II isoforms from these tissues were the first contractile proteins to be studied, members of this class have been referred to as “conventional” (2). This term has set myosin II apart from the other classes of myosins, which in aggregate have been labeled “unconventional.” Structural and enzymatic features that have been thought to be typical of myosin II include the ability to form ordered arrays of thick filaments, the ability of individual motor domains to function independently, rate-limiting phosphate release, and low duty ratio (3, 4). For myosin from contractile tissues like muscle, these features would be essential because they would ensure that the individual heads of a thick filament would spend only a brief time generating force and would not interfere with each other during periods of sustained contraction and shortening.

However, vertebrate myosin II is in fact widely distributed and readily detectable in non-muscle cells as well (5–8). Sequence studies reveal three classes of non-muscle myosin II isoforms named A, B, and C (9, 10). Myosin II is necessary for cell motility, maintenance of normal cell-surface receptor distribution, and stress fiber formation (12–16). Whereas myosin IIA is found in nearly all tissues, the distribution of myosin IIB appears to be more limited (8–10). Myosin IIB comprises ~70% of the myosin II found in the central nervous system and 100% of cardiac non-muscle myosin II. Myosin IIB is required for normal development of the brain and heart (17), and it also plays an essential role in directed growth cone motility (14, 18, 19). In particular, studies of cultured neurons from myosin IIB knockout mice have led to the suggestion that this motor is required for maintenance of cortical tension, a key component in directed cell motility (18).

Non-muscle myosin II thick filaments are 20–30-fold smaller than their smooth or skeletal muscle counterparts, containing <28 myosin molecules (20). This is not surprising because the 1–2-μm long filaments of skeletal or smooth muscle would be far too large to effectively generate force in the presence of the tightly packed meshwork that characterizes the actin cytoskeleton (13). Although the smaller size of non-muscle myosin II-containing thick filaments represents an adaptation to the demands of cytoplasmic contractility, it also presents a challenge for a motor designed to maintain cortical tension. Conventional myosins typically have duty ratios in the range of 3–4%. This low ratio ensures that myosin heads that have completed their power stroke do not resist those heads beginning theirs. For a large thick filament such as myosin II from smooth or skeletal muscle, this does not present a problem because the large number of heads within a thick filament would ensure that at least a few would be strongly attached at any given time, and they would prevent the thick filament from diffusing away. However, a low duty ratio for non-muscle myosin II would mean that there would be an appreciable chance that, at any given time, no heads would be strongly attached to actin. This could be problematic for a myosin designed to generate cortical tension because even brief loss of contact of a myosin IIB filament with actin would lead to immediate loss of tension and elastic recoil of the cytoskeleton.

Given these considerations, we would therefore predict that myosin IIB has a high duty ratio, a feature generally thought to be more characteristic of the unconventional myosins (21, 22). However, a high duty ratio for a filament-forming myosin such as myosin IIB would present another problem: the resistance of bound heads to force generation by actively contracting heads, which has been discussed above. To get around this problem, we would therefore also predict that myosin IIB has an additional feature generally thought to be characteristic of unconventional myosins: a strain-dependent release mechanism (23, 29). In such a mechanism, release of a strongly bound head would occur only when it experienced strain induced by another actively contracting head.

In this study, we have systematically examined the steps that constitute the myosin IIB mechanochemical cycle. This work establishes that, although myosin IIB resembles smooth
muscle myosin II in some aspects of its enzymology, its kinetics also share features that have heretofore been thought to be characteristic of unconventional myosins such as myosins V and VI. This study also provides insight into how the physiologic demand for maintenance of cortical tension is translated into specific changes in the kinetics of the actomyosin ATPase cycle.

MATERIALS AND METHODS

Recombinant Myosin IIB Expression and Purification—To create single-headed, subfragment-1-like myosin IIB constructs, a chicken non-muscle myosin IIB heavy chain cDNA (B1 isoform of loop 1 with no loop 2 insertion; kindly provided by Dr. Robert Adelstein) was truncated at Leu449. A FLAG tag (encoding GYDKDDDDK) was inserted at the C terminus to facilitate purification (24). The recombinant heavy chain protein contains the motor domain and both the essential light chain- and regulatory light chain-binding sites (IQ motifs).

Expression of recombinant myosin IIB subfragment-1 was accomplished via infection of SF9 insect cells with a baculovirus expression vector capable of driving high level expression of foreign proteins. The SF9 cells were co-infected with recombinant virus expressing the myosin IIB heavy chain and with recombinant viruses for the non-muscle essential and regulatory light chains. Details of the protein expression and purification have been published (25). Myosin IIB was made nucleotide-free by incubation in 10 mM EDTA for 20 min at room temperature, followed by gel filtration on Sephadex G-25 (PD-10, Amersham Biosciences).

Fluorescence and Kinetic Methodologies— Steady-state and transient-state fluorescence measurements were made as previously described (25) in samples that had been equilibrated in 20 mM KCl, 25 mM HEPES, 1 mM MgCl2, 1 mM EGTA, and 1 mM dithiothreitol (pH 7.50). Kinetic measurements were made using an Applied Photophysics SX.185 MV stopped-flow spectrophotometer with an instrument dead time of 1.2 ms as described (21, 25, 26).

RESULTS

Our approach in this study was to examine the kinetics of each of the steps in the mechanochemical cycle of myosin IIB. The overall framework that we used is a kinetic scheme that we described for smooth muscle myosin II (26) and that is summarized in Scheme 1, where A is actin, M is myosin, T is ATP, D is ADP, P1 is inorganic phosphate, and weak binding states are in italics and strong binding states are in boldface. The rate constants for these transitions that were determined in this study are summarized in Table I, which includes corresponding values for smooth muscle myosin II and myosin V.

ATP-induced Formation of a Weakly Bound State—ATP binding to actomyosin can be monitored by quenching of a pyrene fluorophore on actin. Formation of a weak binding state occurs as a two-step reaction, with initial formation of a collision complex followed by a first-order quenching of pyrene fluorescence: $\text{AM} + T \rightarrow \text{AM}(T) \rightarrow A^*\text{M}T$; where the asterisk refers to a state of enhanced pyrene fluorescence. Scheme 1 predicts a fluorescence rise that obeys a first-order process whose rate varies hyperbolically with ATP concentration. Fig. 1 confirms this and reveals a maximum rate of 409 ± 13 s⁻¹ and a dissociation constant of 646 ± 41 μM (Table I). Similar results were obtained using the turbidity change at 350 nm as a measure of the dissociation step (data not shown).

ATP Hydrolysis by Myosin IIB—ATP binding to myosin IIB produced an 11% enhancement of tryptophan fluorescence emission, whereas ADP binding produced a 4–5% enhancement (data not shown). Tryptophan fluorescence enhancement produced by ATP binding is characteristic of myosins, and the maximum rate of this process has been shown for both smooth and skeletal muscle myosin II, as well as myosin V, to be a measure of the hydrolysis step, $k_3 + k_{-3}$ (21). As with these other myosins, the rate of this process for myosin IIB varied hyperbolically with ATP concentration (Fig. 2). This dependence defines a maximum rate of 16.7 ± 1.1 s⁻¹ with an apparent ATP dissociation constant of 37 ± 9 μM (Table I).

Phosphate Release—The kinetics of actin-activated phosphate release from myosin IIB were measured in a sequential mixing experiment. Nucleotide-free myosin IIB was mixed with a 10-fold molar excess of ATP. The complex was allowed to age for 500 ms to allow population of the myosin IIB-ADP-P state, and it was then mixed with varying concentrations of actin and 2 mM ADP. ADP was present in the final mixture to block further ATP binding and hydrolysis and to drive the myosin IIB into a strong binding state. Phosphate release was monitored by using MDCC1-labeled phosphate-binding protein at a 10-fold molar excess over active sites (21, 22). The resulting fluorescence enhancement, due to phosphate binding to MDCC-labeled phosphate-binding protein, fit a single exponential process. The rate of this process ($k'_1$) varied linearly over the range of actin concentrations examined (Fig. 3, closed triangles), and the slope of this curve (solid line) defines an apparent second-order rate constant of 0.0038 ± 0.0003 μM⁻¹ s⁻¹.

Because phosphate release is rate-limiting for other myosin II isoforms (27), we expected this to be the case for myosin IIB as well. To test this, we measured the rate of product release from actomyosin IIB as a function of actin concentration by performing the following experiment. Nucleotide-free myosin IIB was mixed with a 10-fold molar excess of 2'-deoxy-mant-ATP. The mixture was allowed to age for 500 ms and then mixed with varying concentrations of actin and 2 mM ADP. As shown in Fig. 3 (open squares), the rate of mant-ADP release also varied linearly with actin concentration (dashed line), but the apparent second-order rate constant is nearly 2.5-fold lower at 0.0015 ± 0.0001 μM⁻¹ s⁻¹. This implies that another step, subsequent to phosphate release, at least partially limits the ATPase rate.

Myosin IIB-ADP and Actomyosin IIB-ADP States—In our previous study of smooth muscle actomyosin, we utilized the fluorescent nucleotide 2’-dmD to examine the sequence of conformational changes that the active site undergoes as it releases ADP (26). Our results could be interpreted in terms of

\[ AM + T \xrightarrow{K_+} AM(T) \xrightarrow{K_-} AM + T \]

\[ AM \cdot D \cdot P_1 \xrightarrow{K_{-3}} AM \cdot D \cdot P_1 \]

\[ AM \cdot D_1 \xrightarrow{k'_1} AM \cdot D_2 \xrightarrow{k_1} AM \]

\[ M \cdot D_1 \xrightarrow{k_2} M \cdot D_2 \xrightarrow{k_1} M \]

**Scheme 1**

**Scheme 2**

1 The abbreviations used are: MDCC, N-[2-(1-maleimidylethyl)-7-(diethylamino)coumarin 3-carboxamide; mant-, N-methylanthraniloyl; 2’-dmD, 2’-deoxy-N-methylanthraniloyl-ADP.
Table I
Rate and equilibrium constants for actomyosin IIB, smooth muscle actomyosin II, and actomyosin V

The conditions were as follows: 20 mM KCl, 25 mM HEPES, 2 mM MgCl₂, 1 mM EGTA, and 1 mM dithiothreitol (pH 7.50) at 20 °C.

| Parameter | Actomyosin IIB | Smooth muscle actomyosin IIa | Actomyosin Vb |
|-----------|---------------|---------------------------|--------------|
| kₐ (binding of rigor myosin to actin) | 1.40 μM⁻¹ s⁻¹ | 1.24 μM⁻¹ s⁻¹ | 73 μM⁻¹ s⁻¹ |
| k₆'K₆' (ATP binding) | 6.83 μM⁻¹ s⁻¹ | 0.47 μM⁻¹ s⁻¹ | 0.9-1.6 μM⁻¹ s⁻¹ |
| k₋₋ (ATP-induced dissociation) | 409 ± 13 s⁻¹ | 1300 s⁻¹ | 876 s⁻¹ |
| k₋ + k₋₋ (ATP hydrolysis) | 16.7 ± 1.1 s⁻¹ | 25 s⁻¹ | 750 s⁻¹ |
| k₋₋ (phosphate release) | 3.7 s⁻¹ | 3.0 s⁻¹ | >250 s⁻¹ |
| k₋ (weak-to-strong isomerization) | 6.4 ± 0.6 s⁻¹ | 6.9 s⁻¹ | |
| k₋₋₋ (ADP isomerization) | 0.8 s⁻¹ | 30 s⁻¹ | |
| k₋₋ (ADP dissociation) | 7.6 ± 1.4 s⁻¹ | 45 s⁻¹ | 12-16 s⁻¹ |
| k₋₋₋ (ADP isomerization) | 0.6 s⁻¹ | 1.0 ± 0.03 s⁻¹ | 12-15 s⁻¹ |
| K_BADP, (ADP affinity) | 0.15 μM | 4.7-5.3 μM | 0.70-0.93 μM |
| Duty ratio | 0.82 ± 0.18 | 0.03-0.04 | 0.7 |

a Data from Refs. 26 and 27.

b Calculated from the model presented in Scheme 1.

d(ADP) (ADP affinity) 0.15

d(ADP) (ADP dissociation) 7.6

d(ADP) (ADP isomerization) 0.8 s⁻¹

d(ADP) (phosphate release) 3.7 s⁻¹

d(ADP) (ATP binding) 0.63

d(ADP) (ATP-induced dissociation) 409

d(ADP) (weak-to-strong isomerization) 6.4

d(ADP) (ATP hydrolysis) 16.7

d(ADP) (phosphate release) 3.7

d(ADP) (ATP binding) 0.63

d(ADP) (ATP-induced dissociation) 409

d(ADP) (weak-to-strong isomerization) 6.4

d(ADP) (ATP hydrolysis) 16.7

d(ADP) (phosphate release) 3.7

FIG. 1. Kinetics of ATP-induced population of the weak binding state. Varying concentrations of ATP were mixed in the stopped-flow spectrometer with a complex of 2 μM myosin IIB and 5 μM pyrene-labeled actin. The resulting fluorescence increase fit a single exponential process (inset), and a plot of rate versus ATP concentration fit a rectangular hyperbola. The conditions were as follows: 20 mM KCl, 25 mM HEPES, 2 mM MgCl₂, 1 mM EGTA, and 1 mM dithiothreitol (pH 7.50) at 20 °C.

FIG. 2. Kinetics of ATP-induced enhancement of myosin IIB tryptophan fluorescence emission. Nucleotide-free myosin IIB (5 μM) was mixed with varying concentrations of ATP in the stopped-flow spectrometer, and the reaction was monitored by the enhancement of tryptophan fluorescence emission (inset). The rate varied hyperbolically with [ATP]; and the maximum rate, which is a measure of the hydrolysis step, extrapolated to 16.7 ± 1.1 s⁻¹. The conditions were as described in the legend to Fig. 1.
Phosphate release was measured in a sequential mixing stopped-flow spectrometer with a 10-fold molar excess of ATP. The mixture was allowed to age for 500 ms and then mixed with varying concentrations of actin and 2 mM ADP. Each of the curves had a 10-fold molar excess of MDCC-labeled phosphate-binding protein over the final myosin IIB concentration. Phosphate release, as monitored by MDCC-labeled phosphate-binding protein fluorescence increase, occurred as a first-order process whose rate (open triangles) varied linearly with actin concentration. This is compared with the rate of mant-phosphate release in a single turnover assay. This was measured by mixing 2 μM myosin IIB with 20 μM 2′-deoxy-ATP in the sequential mixing stopped-flow spectrometer, aging for 500 ms, and mixing again with actin and 2 mM ADP. The resulting fluorescence decrease fitted a single exponential decay, and the rate (open squares) is plotted versus actin concentration. The conditions were as described in the legend to Fig. 1.

Thus, the rapid phase is due to pyrene quenching by one or more strong myosin IIB-ADP binding states.

By contrast, the slow phase demonstrates a hyperbolic dependence on actin concentration (open squares, solid line), defining a maximum rate of 6.4 ± 0.6 s⁻¹ and an apparent dissociation constant of 5.9 ± 1.5 μM. These kinetics are to be expected for a two-step binding reaction, with formation of an initial weak actin-binding complex followed by isomerization to a strong binding complex that quenches the pyrene fluorescence: A → M · D₁ ⇌ A · M · D₂ → A · M. Thus, in the absence of actin, myosin IIB-ADP is a mixture of strong and weak actin binding and strong and weak ADP binding states. However, actin binding shifts this distribution to produce a mixture of strong and weak ADP binding states that uniformly bind strongly to actin. A similar situation was found to apply to smooth muscle myosin II (26), which could be explained by Scheme 3, where Kₗ, Kₛ, and Kₜ are affinity constants for the various myosin IIB-ADP states for actin. This scheme allows us to integrate the fluorescence lifetime and pyrene kinetic studies. Because ADP does not quench the fluorescence of pyrene-labeled actomyosin IIB, we conclude that the M · D₁ state is not populated (e.g., Kₚ' > 1); and therefore, f₂/f₁ = [A · M · D₁]/[A · M · D₂] = Kₛ'. Furthermore, the presence of two phases in the pyrene fluorescence transient (Fig. 5) implies that, like smooth muscle myosin, an appreciable population of myosin IIB-ADP is in the M · D₁ state. Consequently, Kₗ' ≪ Kₛ, which in turn requires that Kₗ ≪ Kₛ'. This would be expected because, by definition, the actin affinity of the M · D₁ state should be much less than that of the M · D₂ state.

**Kinetics of ADP Binding to and Release from Actomyosin II**—Scheme 3 predicts that ADP dissociation from actomyosin should occur in two phases, reflecting the AM · D₁ → A · M · D₂ → AM transitions. This prediction is confirmed in Fig. 6A. Mixing
myosin IIB–2’dmD with actin and 4 mM ADP produced a biphasic fluorescence decrease. The rate of the faster phase shows a hyperbolic dependence on actin concentration, with an extrapolated value of $8.2 \pm 1.9 \text{ s}^{-1}$ and an apparent dissociation constant of $10.2 \pm 4.9 \text{ mM}$, whereas that of the slower phase shows little actin concentration dependence ($0.64 \pm 0.06 \text{ s}^{-1}$). The rates of these two phases depend on the values of $k_{-7}$, $k_{-6}$, and $k_{7}$.

To measure $k_{7}$, we examined the kinetics of the fluorescence enhancement produced by mixing nucleotide-free actomyosin IIB with 2’-dmD. A plot of rate versus 2’-dmD concentration should be linear, and Fig. 6B confirms this. The slope defines an apparent second-order rate constant of $4.90 \pm 0.05 \text{ s}^{-1} \text{ mM}^{-1}$; the y intercept defines a $k_{7}$ value of $7.5 \pm 1.4 \text{ s}^{-1} \text{ mM}^{-1}$; and the ratio defines a $K_7$ value of $1.56 \text{ mM}$ (Table I).

**ADP Affinity for Actomyosin IIB**—When interpreted in the context of Scheme 3, our results indicate that $K_7$ for actomyosin IIB is ~11-fold lower than for smooth muscle actomyosin II (26). This in turn implies that the ADP affinity for actomyosin IIB should be correspondingly higher. We measured the affinity of ADP for actomyosin IIB by incubating a complex of 300 nM nucleotide-free myosin IIB and 300 nM pyrene-labeled actin with varying concentrations of ADP and mixing in the stopped-flow spectrometer with 4 mM ATP. The resulting fluorescence decrease consisted of two phases: a rapid phase (rate of $250–350 \text{ s}^{-1}$) due to ATP binding to nucleotide-free actomyosin IIB and a slow phase (rate of $0.71 \pm 0.08 \text{ s}^{-1}$) due to rate-limiting ADP release followed by ATP binding and dissociation (Fig. 7A). The total amplitude remained constant over the range of ADP concentrations tested, implying little ADP-induced dissociation (data not shown). However, the relative amplitudes of these two phases did vary with ADP concentration in a reciprocal manner and are plotted versus ADP concentration. Data were fit to Equation 1 for $\theta$ (dashed curve) and $1 - \theta$ (solid curve). For both curves, the midpoints define an apparent ADP dissociation constant of 154 nM. The conditions were as described in the legend to Fig. 1.

$$\theta = \frac{K_{7} \cdot [ADP]}{K_{7} \cdot [ADP] + [K_{7}^{-1} + 1]} \quad \text{(Eq. 1)}$$

and fitting to this equation therefore allows us to independently test our model. This was accomplished by using the value of $K_7$ determined from Fig. 6B, yielding a $K_7$ value of 0.14 at 20 °C. This is in excellent agreement with the value of 0.12 that we had determined from the fluorescence lifetime data (Fig. 4).

**Fig. 6.** Kinetics of 2’-dmD release from (A) and binding to (B) actomyosin IIB. A, 2 μM myosin IIB and 10 μM 2’-dmD were mixed in the stopped-flow spectrometer with varying concentrations of actin and 2 mM ADP, and fluorescence was monitored by fluorescence resonance energy transfer from myosin IIB tryptophan residues to the m-fluorophore. The resulting fluorescence decrease (inset) fit a double exponential process. The rate of the faster phase varied hyperbolically with actin concentration, whereas that of the slower phase shows little actin concentration dependence ($0.64 \pm 0.06 \text{ s}^{-1}$). The rates of both phases remained relatively constant over the range of actin concentrations tested. B, a complex of 2 μM myosin IIB and 10 μM actin was mixed in the stopped-flow spectrometer with varying concentrations of 2’-dmD. The resulting fluorescence increase (inset) fit a single exponential rise with a linear dependence of rate on [2’-dmD] (solid line). The conditions were as described in the legend to Fig. 1.

**Fig. 7.** ADP affinity for actomyosin IIB. A, varying concentrations of ADP were added to nucleotide-free myosin IIB and pyrene-labeled actin, followed by mixing in the stopped-flow spectrometer with 4 mM ATP. The resulting fluorescence transient consisted of a fast phase with a rate of $250–350 \text{ s}^{-1}$ (inset) and a slower phase with a rate of $0.71 \pm 0.08 \text{ s}^{-1}$. The rates of both phases remained relatively constant over the range of ADP concentrations tested. B, shown is a plot of the fractional amplitudes of the fast (closed squares) and slow (open triangles) phases. These amplitudes varied reciprocally and are plotted versus ADP concentration. Data were fit to Equation 1 for $\theta$ (dashed curve) and $1 - \theta$ (solid curve). For both curves, the midpoints define an apparent ADP dissociation constant of 154 nM. The conditions were as described in the legend to Fig. 1.
the midpoints of the curves in Fig. 7B define an apparent ADP dissociation constant of 154 nM (Table I). This compares with a value of 5 μM for smooth muscle actomyosin II (30). Myosin IIB Duty Ratio—The duty ratios of smooth and skeletal muscle myosin II are in the range of 3–4% because phosphate release is at least 10-fold slower than ADP release (26, 27). However, the slow phase of the 2’-dmD release transient (Fig. 6A), which is a measure of the rate of the AM-D1 → AM-D2 transition, is no faster than phosphate release (Fig. 3). We would therefore expect that the duty ratio of myosin IIB should be appreciably higher than that of muscle myosin II isofroms. We directly measured the duty ratio as a function of actin concentration by measuring the fluorescence of a complex of myosin IIB and a 5-fold molar excess of pyrene-labeled actin in the presence and absence of 4 μM ATP. We compared these results with those obtained with an equimolar concentration of pyrene-labeled actin without myosin to calculate the fraction of myosin strongly bound. Fig. 8 depicts the data, which fit a hyperbola and extrapolate to a maximum duty ratio of 0.82 ± 0.16.

DISCUSSION

The underlying premise of this work is that the physiologic demands placed on a motor shape its enzymology and, conversely, that understanding the enzymology of a motor sheds light on its physiologic role. Our study of the mechanochemical cycle of the myosin IIB ATPase illustrates this concept. The key finding of this study is that isomerization of actomyosin IIB-ADP states is the rate-limiting step, and this has profound effects on the behavior of this motor.

Isomerization of Myosin IIB-ADP States—As with smooth muscle myosin II, myosin IIB-ADP is a mixture of several states, both in the presence and absence of actin. Measurements of the fluorescence lifetimes of bound 2’-dmD, of the kinetics of myosin IIB-ADP binding to pyrene-labeled actin, of the kinetics of 2’-dmD binding to and release from actomyosin IIB, of the ADP affinity for actomyosin IIB, and of the myosin IIB duty ratio are all consistent with a general scheme first described in our study of smooth muscle actomyosin (26) and presented in this study as Scheme 3.

Interpretation of the data for actomyosin IIB within the context of this scheme is fairly straightforward because the AM-D1 state is not populated at equilibrium. This allows us to utilize the fluorescence lifetime data to directly calculate a value of $K_D'$. The validity of our model is further supported by our fitting data from Fig. 7B to Equation 1, which provides an independent measure of $K_D'$ and one that is remarkably close to that generated from the lifetime data. This analysis allows us to make direct comparisons with smooth muscle actomyosin II. In doing so, it becomes apparent that a major difference between these two contractile systems is in the value of $K_D'$, which is 11-fold higher for smooth muscle actomyosin. This difference would favor formation of the AM-D2 state for actomyosin IIB, and it would consequently slow ADP release and enhance relative ADP affinity compared with smooth muscle actomyosin. In fact, the affinity of ADP for actomyosin IIB is higher than for any other myosin isoform studied (Table I). This finding also explains why our measurements of the steady-state ATPase of actomyosin IIB demonstrated no evidence of actin activation (data not shown) because sufficient ADP would be generated within a few turnovers to effectively inhibit the enzyme.

Determining the corresponding equilibrium constants for myosin IIB-ADP is less straightforward because our data indicate that the M-D2, M-D1, and M-D3 states are all populated at equilibrium. An assumption underlying the formulation of Scheme 3 is that actin affinity and nucleotide affinity are partially uncoupled in the presence of ADP, which means that $K_a = K_s$. Furthermore, for myosin IIB-2’-dmD at 20 °C, $f_2/f_1 = [M-D_2]/([M-D_1] + [M-D_2]) = 0.58$. Combining these two constraints gives us $K_a$ and $K_s$ estimates of 1.5 and 0.5, respectively, consistent as well with the requirement that $K_s' > K_a$.

What is the pathway that actomyosin IIB utilizes in its mechanochemical cycle? Given that $K_s'$ is very high and the DM-D1 state is not populated in the steady state, it is reasonable to assume that $k_d$ is also high. Therefore, the main pathway following rebinding of myosin IIB-ADP to actin would be

\[ \text{AM-DP}_i \rightarrow \text{DM-D}_2 \rightarrow \text{AM-D}_2 \rightarrow \text{AM-D}_3. \]

This in turn implies that myosin IIB bypasses the M-D1 state during its normal mechanochemical cycle. This conclusion is consistent with a recent report that the lever arm in smooth muscle myosin II-ADP (which we have shown is >95% in the M-D1 state) (26) assumes a rigor-like orientation in the absence of actin (11). These results predict that binding of M-D2 to actin would rotate the lever arm from a rigor position to an “ADP”-like position, which would oppose the normal physiologic operation of this motor.

The kinetics of 2’-dmD release from myosin IIB followed a single exponential process at 20 °C, which could be explained if $K_a$ and $K_s$ were rapid equilibria relative to $k_d$. For 2’-dmD release from actomyosin IIB, two phases were seen. Solution of the rate equations is as described in our prior study of smooth muscle actomyosin II (26): $\lambda_{1,2} = (S_2\sqrt{S^2 - 4C})/2$, where $S = k_1' + k_0' - k_{-1}'$ and $C = k_0'k_{-1}'$. This solution must be consistent with the value of $K_s'$ derived from lifetime measurements and the value of $k_{-1}'$ derived from the kinetics of 2’-dmD binding to actomyosin IIB. A reasonable fit that satisfies these requirements is obtained with $k_1' = 0.8 s^{-1}, k_{-1}' = 4 s^{-1}$, and $K_s' = 7.0 s^{-1}$. The corresponding values for smooth muscle actomyosin are $k_1' = 30 s^{-1}, k_{-1}' = 15 s^{-1}$, and $K_s' = 45 s^{-1}$ (Table I). Whereas each of these rate constants is considerably lower for myosin IIB, the greatest decrease is seen in the value of $k_{-1}'$, which assumes a value that is slower than the rate of phosphate release by other myosin isoforms (Table I).

Duty Ratio and Rate-limiting Step—If $k_{-1}'$ is the rate-limiting step in the mechanochemical cycle of actomyosin IIB, then the duty ratio should be appreciably higher than in other myosin II isoforms. We confirmed this (Fig. 8) and found that the duty ratio was in the same range as that seen in some unconventional myosins such as myosin V. The value of the duty ratio varied with actin concentration, and its maximum value is related to two kinetic steps, the rate of formation of a strong
binding state and the effective rate of ADP release: duty ratio = (rate of formation of AM-D) / (rate of formation of AM-D) + rate of loss of AM-D1 = k4/(k4 + k5). Inserting the values of k4 and the duty ratio yields a calculated k4 value of 3.7 s⁻¹, which confirms that for myosin IIB, the rate-limiting step is not phosphate release, but rather the AM-D1 → AM-D2 transition.

Comparison with Other Myosins—The enzymology of smooth muscle myosin II has served as a standard for conventional myosin II behavior. As summarized in Table I, this behavior includes rapid induction of the weak binding conformation by ATP, hydrolysis at −25 s⁻¹, rate-limiting phosphate release, actin-activated ADP release, and a low duty ratio. These are the characteristics expected of a motor that operates in a large ensemble and that is designed to produce shortening. By contrast, the physiologic demands on an unconventional myosin that acts in isolation, such as myosin V, predicts that it would need a high duty ratio and a mechanism to allosterically regulate the timing of forward stepping so that the two heads of such a motor would work in a coordinated fashion. Myosin V appears to have addressed this requirement by markedly shortening the lifetime of weakly bound states (Table I). Thus, ATP hydrolysis and phosphate release are accelerated up to several hundredfold, whereas ADP release kinetics remain similar to those for smooth muscle myosin II. The net effect is that ADP release is rate-limiting and the duty cycle is 20–25-fold larger.

A variety of inhibition studies have led to the suggestion that myosin IIB generates sustained cortical tension and works in small ensembles of <30 motors per filament. These physiologic demands would be expected to have specific effects on the enzymology of this motor. We would predict that myosin IIB is a relatively slow enzyme because rapid shortening should not be required of a motor designed to produce sustained tension. Furthermore, we would expect a high duty ratio and a strain-dependent release mechanism, which would ensure that heads would remain strongly attached for a relatively long duration and would not release until other heads of the same filament were strongly attached.

The data presented in this study support these predictions. Like its unconventional relative myosin V, myosin IIB has arranged its enzymology to ensure that it has a high duty ratio. As noted above, the value of the duty ratio is determined by the relative rates of phosphate and ADP release. The high duty ratio of myosin V results from its rapid rate of phosphate release and relatively slow (compared with smooth or skeletal muscle myosin II) rate of ADP release (Table I). By contrast, myosin IIB has engineered a high duty ratio in a different manner. Its rate of phosphate release is nearly identical to that for smooth muscle myosin II. However, ADP release is markedly slow because of a rate-limiting isomerization of ADP states (k₄). This produces a slowly cycling motor that resembles myosin V in some aspects and smooth muscle myosin II in others. Furthermore, myosin IIB has accomplished this largely by altering one step: the isomerization of actomyosin IIB-ADP states.

Finally, reducing the value of k₄ has the effect of markedly enhancing ADP affinity. Because intracellular ADP concentrations are 12–50 μM (28), the myosin IIB motor would be permanently stalled unless an additional mechanism existed to accelerate ADP release. Such a mechanism could involve mechanical strain, which has been used to explain the processive movement of myosin V (23). In myosin V, strain produced by attachment of the head to actin has been proposed to accelerate ADP release from the trailing head. We argue that a similar situation applies as well to myosin IIB, although in this case, the strain applied to one head may originate from other heads of other myosin IIB molecules that occupy the same thick filament. Furthermore, although strain may accelerate ADP release from native myosin V, single-headed constructs are still capable of releasing nucleotide (21). Hence, strain modulates the cycle, but is not essential for myosin V to be enzymatically active. By contrast, the very high ADP affinity of actomyosin IIB means that a strain-dependent release mechanism is an absolute requirement for myosin IIB to function as an enzyme and motor.

Conclusion—This study has shown that myosin IIB, a member of the conventional myosin II family, has a number of features that it shares with unconventional myosins such as myosins V and VI. These include a high ADP affinity, a high duty ratio, rate-limiting ADP release, and the need for a strain-dependent mechanism to ensure normal activity. Each of these features would help support the putative role of this motor as a generator of cortical tension and, taken together, demonstrate how the physiologic demands placed on this motor make it unconventionally conventional.

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