Dictyostelium Myosin-5b Is a Conditional Processive Motor*  

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Dictyostelium myosin-5b is the gene product of myoJ and one of two closely related myosin-5 isoenzymes produced in Dictyostelium discoideum. Here we report a detailed investigation of the kinetic and functional properties of the protein. In standard assay buffer conditions, Dictyostelium myosin-5b displays high actin affinity in the presence of ADP, fast ATP hydrolysis, and a high steady-state ATPase activity in the presence of actin that is rate limited by ADP release. These properties are typical for a processive motor that can move over long distances along actin filaments without dissociating. Our results show that a physiological decrease in the concentration of free Mg\(^{2+}\)-ions leads to an increased rate of ADP release and shortening of the fraction of time the motor spends in the strong actin binding states. Consistently, the ability of the motor to efficiently translocate actin filaments at very low surface densities decreases with decreasing concentrations of free Mg\(^{2+}\)-ions. In addition, we provide evidence that the observed changes in Dictyostelium myosin-5b motor activity are of physiological relevance and propose a mechanism by which this molecular motor can switch between processive and non-processive movement.

Class 5 myosins are dimeric actin-based motors that are involved in various forms of intracellular trafficking (1). Depending on the isoform and cell type, class 5 myosins have been implicated in the movement of membranes and organelles (2–4), the transport of synaptic and secretory vesicles (5, 6), and the active delivery of receptors and mRNA-protein complexes to their place of action (7, 8). The unique modular structure of class 5 myosins is essential for these specialized transport functions (9, 10). Each heavy chain of a dimeric myosin-5 molecule consists of a motor domain that binds actin and hydrolyzes ATP (11), followed by a long neck region to which up to six light chains can bind (12, 13). Parts of the adjacent tail region form a coiled-coil and the C terminus consists of a globular domain that mediates the binding to cargo and regulates activity of the motor (14, 15).

Despite the high sequence similarity between myosin-5 isoforms, the individual members display differences in their mechanoenzymatic properties, which characterize them either as processive or non-processive motors. Processive myosins, like vertebrate myosin-5a, are capable of taking successive steps along actin as single molecules before detaching (16). The overall movement has been described as a coordinated stepping process of both heads in a hand-over-hand mechanism that is driven by intramolecular strain (17, 18). In contrast, non-processive myosins bind to the actin filament perform just one step and then dissociate rapidly. A notable difference between processive and non-processive myosins is displayed in the duty ratio, i.e. the fraction of the total ATPase cycle time a motor spends in the strong actin binding states. Processive myosins have a high duty ratio (>0.5), whereas non-processive myosins display a low duty ratio that is generally far below 0.5. Characteristic kinetic parameters contributing to a high duty ratio thus minimizing early detachment from actin include (i) a fast ATP hydrolysis rate, (ii) a high affinity for actin in the weak binding states, (iii) a high ADP affinity in the actin-bound states, (iv) a rate-limiting ADP dissociation rate, (v) an increased P\(_i\) release rate, and (vi) a weak coupling between nucleotide and actin binding sites.

The relevance of a high duty ratio for processive movement has been shown by comparison of the kinetic properties of class 5 myosins from different subclasses and organisms. Accordingly, vertebrate myosin-5a is a high duty ratio motor that moves processively along actin filaments (19, 20). Recently, a kinetic study of human myosin-5b revealed that this myosin is also characterized by a high duty ratio; however, direct observation of the predicted processivity has not been reported (21). Not all class 5 myosins are high duty ratio motors. Homo sapiens myosin-5c (22, 23), Drosophila melanogaster myosin-5 (24), and Saccharomyces cerevisiae myo2p and myo4p (25, 26) display properties that are not compatible with those of a processive motor. It is assumed that these myosins need to function as ensembles for the efficient intracellular translocation of cargo. So far, there is limited information about the kinetic, structural, and mechanoenzymatic properties of class 5 myosins that belong to subclasses other than subclass 5a. Thus, it is difficult to define in detail the parameters and molecular mechanisms that distinguish processive class 5 myosins from non-processive ones. The members of the respective groups are assumed to use different ways to couple conformational changes at the nucleotide binding regions to changes that occur at the actin binding sites during the ATPase cycle.

This study provides a detailed kinetic and functional characterization of Dictyostelium discoideum myosin-5b (Dd myoJ) in comparison with vertebrate myosin-5a and myosin-5c. The results presented in the following manuscript show that Dictyostelium myosin-5b is a processive motor with a high duty ratio that is close to that of vertebrate myosin-5a.
myosin-5b), a heavy chain dimer forming class 5 myosin, which previously has been referred to as Myoj (27, 28). We compare the results obtained for Dd myosin-5b with those previously reported for processive and non-processive members of the myosin-5 family (19, 21–24). Our investigations reveal that under standard assay conditions the kinetic properties of Dd myosin-5b are similar to those of other processive myosins: ADP-release limiting the actomyosin ATPase cycle, a low degree of coupling between the nucleotide and actin binding sites, and a high duty ratio. We show that changes in the concentrations of free Mg\(^{2+}\)-ions that lie in the physiological range modulate the ADP release kinetics of the motor and affect the duty ratio, which is a critical determinant for processivity. Our results show that this particular mechanism enables native Dd myosin-5b to switch between processive and non-processive motor activity in the context of the contractile vacuole.

**EXPERIMENTAL PROCEDURES**

Reagents—Standard chemicals, TRITC-phalloidin, and anti-His antibody were purchased from Sigma; restriction enzymes, polymerases, and DNA-modifying enzymes were purchased from MBI-Fermentas and Roche Applied Sciences.

Plasmid Construction—The oligonucleotides 5’-C GGA TCC ACC ACA TCA ACA ATT-3’ and 5’-GT CTC GAG CAC TAC GAT CCA-3’ were used to isolate a PCR fragment from *Dictyostelium* AX2 genomic DNA that encodes the 829 amino acids of the motor domain of *Dictyostelium* myosin-5b (28). The product was cloned into the expression vector pDXA-EYFP (29). All plasmids were confirmed by sequencing.

Protein Production and Purification—Plasmids for the high level production of the *Dd* myosin-5b gene were amplified by PCR from genomic DNA and inserted into the pDXA-J829 expression vector using XhoI as a unique restriction site. This produced the plasmid pDXA-Drd myosin-5b encoding the full-length protein. It was digested with BamHI and SphI and the gene fragment was cloned into the vector pDXA-EYFP-MCS for the N-terminal fusion with EYFP (29). All plasmids were confirmed by sequencing.

**Procesive Dictyostelium Myosin-5b**

The **Scheme 1. Kinetic reaction scheme of the actomyosin ATPase cycle.** A refers to actin, *M* to myosin, *T* to ATP, and *D* refers to ADP. Rate constants are referred to as *k* *\(_{M,1}\)* and *k* *\(_{-M,1}\)*, assigned to the corresponding forward and reverse reactions. An additional notation is used that distinguishes between the constants in the absence and presence of actin by italic type (*k* *\(_{M,1}\)\(_{i}\)* and *k* *\(_{-M,1}\)*, respectively; subscript *A* refers to actin (*k* *\(_{A}\)* and subscripts *D* (*k* *\(_{D}\)* refers to ADP.

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Kinetic Measurements—ATPase activities were measured at 25°C with the NADH-coupled assay as described previously (36). Values for *k* *\(_{cat}\)* and *k* *\(_{app}\)* were calculated from fitting the data to the Michaelis-Menten equation. Transient kinetic experiments were performed at 20°C with either a Hi-tech Scientific SF-61 DX single mixing stopped-flow system or an Applied Photophysics PiStar 180 Instrument in MOPS buffer (25 mM MOPS, 100 mM KCl, 1 mM dithiothreitol, pH 7.0) supplemented with varying concentrations of MgCl\(_2\) using procedures and kinetic models described previously (37). Free Mg\(^{2+}\)-ion concentrations were calculated using Maxchelator software as described (38). Kinetic parameters of nucleotide and actin interactions were analyzed in terms of the model shown in Scheme 1.

Direct Functional Assays—Actin-sliding motility was measured as described previously (30). The movement of more than 200 TRITC-phallolidin-labeled actin filaments was recorded for each individual concentration of free Mg\(^{2+}\)-ions. Automated actin filament tracking was performed with the program DiaTrack 3.01 (Semasoph, Switzerland) and data analysis was performed with Origin 7.0 (Originlab, USA).

Landing assays were performed as described by Rock et al. (39) with the following modifications: *Dd* myosin-5b molecules were immobilized on nitrocellulose-coated coverslips via anti-penta-His antibodies (concentration range 0.5 to 41 μg/ml) to obtain surface densities between 50 and 4000 myosin molecules/μm\(^2\). The assay was started by the addition of TRITC-phallolidin-labeled actin (100 nm) to the motility buffer (described above) containing 1.5 mM Mg\(^{2+}\)-ATP and varying concentrations of free Mg\(^{2+}\)-ions. The landing events were recorded with an objective type TIRF microscope equipped with a 532 nm diode laser (150 milliwatts). An inverted micro-
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| Myosin        | Basal ATPase | Michaelis-Menten parameters | kcat/KA | Activation |
|---------------|--------------|-----------------------------|---------|------------|
| Dd myosin-5b  | 0.069 ± 0.01 | 12.4 ± 0.6                  | 21 ± 2  | 0.59 ± 0.12 | 179 ± 12 |
| Hs myosin-5b  | 0.09         | 9.7                        | 8.5     | 1.14       | 107      |
| Gg myosin-5d  | 0.03         | 15                        | 1.4     | 11         | 500      |
| Hs myosin-5c  | 0.1/0.05     | 6.5/1.8                    | 62/42.5 | 0.1/0.042  | 64/35    |
| Dm myosin-5b  | 0.1          | 12.5                       | 9.9     | 1.26       | 125      |

*Values for kcat and KA were calculated from fitting the data to the Michaelis-Menten equation.

ATP BINDING TO Dd MYOSIN-5b AND ATP-INDUCED DISSOCIATION OF ACTO-DD MYOSIN-5b—ATP binding to the Dd myosin-5b motor domain was monitored from the increase in intrinsic protein fluorescence following the addition of ATP. Fluorescence transients were best fit to single exponentials at all ATP concentrations examined. In the range from 5 to 25 μM ATP, the observed rate constants were linearly dependent upon ATP concentration. The apparent second-order rate constant obtained from the slope corresponds to kcat/KA+1 = 0.47 μM−1 s−1. At higher ATP concentrations the observed rate constants kobs followed a hyperbolic dependence (Fig. 1A, filled circles). At saturating ATP concentrations, kmax defines the maximum rate of the conformational change that corresponds to the rate of ATP hydrolysis in the absence of actin (k+3 + k−3). In the case of Dd myosin-5b this rate is >300 s−1 (Table 2).

ATP binding to acto-DD myosin-5b was followed by observing the exponential increase in fluorescence of pyrene-actin as the actomyosin complex dissociates following the addition of excess ATP. The mechanism of ATP-induced fluorescence enhancement was modeled according to Scheme 2, which describes a two-step mechanism for ATP binding to actomyosin.

\[ \text{ATP} + \text{M} \rightarrow \text{ATP-M} \]

\[ \text{ATP-M} \rightarrow \text{ATP-M} \rightarrow \text{M} \]

\[ \text{ATP-M} \rightarrow \text{M} \]

**RESULTS**

All kinetic experiments were performed with a single-headed Dd myosin-5b construct (I829) comprising 829 amino acids of the motor domain. Nucleotide and actin interactions were analyzed according to Scheme 1.

**Steady-state ATPase Activity of Dd Myosin-5b**—The steady-state ATPase activity of Dd myosin-5b was measured in the absence and presence of actin in the range from 0 to 60 μM actin. Dd myosin-5b displays a basal ATPase rate (kbasal) of 0.069 s−1. The maximum actin-activated ATPase activity (kcat) is 12.4 s−1 and comparable with the steady-state ATPase rates reported for other class 5 myosins. Half-maximal activation of the ATPase (Kapp) is reached at 21 μM F-actin and the apparent second-order rate constant for actin binding (kcat/Kapp) is 0.59.

The obtained steady-state parameters are summarized in Table 1, together with published values of human myosin-5b (H. sapiens myosin-5b), chicken myosin-5a (Gallus gallus myosin-5a), human myosin-5c (H. sapiens myosin-5c), and Drosophila myosin-5 (Dm myosin-5).

**AdP Binding to Dd Myosin-5b in the Presence and Absence of Actin**—Because binding of ADP to the motor domain of Dd myosin-5b did not result in a change of the fluorescence signal, neither in the absence nor presence of F-actin measurements were performed using the fluorescent analogue mantADP. Binding of mantADP was determined by monitoring the increase in mant-fluorescence upon the addition of ATP. The concentration of the fluorescent analogue to the Dd myosin-5b construct was 0.12 μM, and the concentration of ATP was 0.01 μM. The observed rate constants were determined from the slopes of the straight lines fitted to the data. The ratio of kcat/KA Admirable
actin to the Dd myosin-5b motor domain. The observed rate constants were linearly dependent upon F-actin concentration over the range studied (Fig. 1C, filled circles). The data were modeled as simple bimolecular reactions. The apparent second-order rate constant of pyrene-actin binding ($k_{+A}$) was obtained from the slope of the plot giving a value for $k_{+A}$ of 1.17 $\mu M^{-1}$ s$^{-1}$. The presence of 1 mM ADP did not significantly affect the second-order rate binding constant ($k_{+DA}$) to F-actin to Dd myosin-5b (Fig. 1C, filled squares).

Pyrene-actin dissociation from Dd myosin-5b was measured by competition with F-actin after mixing an equilibrated mixture of pyrene-acto-Dd myosin-5b with a 40-fold excess of unlabeled F-actin. In the absence and presence of ADP, the observed processes could be fit to single exponentials where $k_{obs}$ corresponds directly to $k_{-A}$ and $k_{-DA}$, respectively. The rates of actin displacement in the absence and presence of 1 mM ADP ($k_{-A} = 0.023 \pm 0.001 \mu M^{-1}$ s$^{-1}$ and $k_{-DA} = 0.03 \pm 0.001$ s$^{-1}$) are very similar and indicate that the tight association of F-actin to Dd myosin-5b is not affected by the presence of excess amounts of ADP. Furthermore, the apparent acto-Dd myosin-5b affinities ($K_A$ and $K_{DA}$) were determined from the ratio of the rate constants for actin binding and dissociation. The parameters are summarized in Table 2.

**ADP Dissociation from Dd Myosin-5b and Acto-Dd Myosin-5b**—The rate of ADP dissociation was determined by monitoring the decrease in fluorescence upon displacement of mantADP from the myosin-mantADP and actomyosin-mantADP complex by the addition of excess ADP. The observed processes could be fitted to single exponentials where $k_{obs}$ corresponds directly to the ADP dissociation rates $k_{-D}$ in the absence and $k_{-AD}$ in the presence of actin (Scheme 1). MantADP dissociation from Dd myosin-5b was ~25-fold increased by actin from $k_{-D} = 0.92$ s$^{-1}$ to $k_{-AD} = 21.6$ s$^{-1}$ (Table 2).

**ADP Affinity of Dd Myosin-5b in the Absence and Presence of Actin**—The affinity of ADP for Dd myosin-5b was determined by monitoring the reduction in the rate of pyrene-actin binding to the myosin motor domain as a function of ADP concentration. The decrease in pyrene fluorescence followed single exponentials at all ADP concentrations examined. The $k_{obs}$ values plotted against the ADP concentration are shown in Fig. 2A. High ADP concentrations decreased the observed rate of pyrene-actin binding ~2-fold. Fitting the data to a hyperbola gives an affinity constant of ADP for Dd myosin-5b ($K_A$) of 5.5 $\mu M$. This value is consistent with the calculated affinity constant $K_A = 5.4 \mu M$ obtained from $k_{-D}/k_{+D}$.

The affinity of ADP for the actomyosin complex ($K_{AD}$) was determined from the inhibition of the ATP-induced dissociation of acto-Dd myosin-5b by ADP. The observed rate of actin dissociation from Dd myosin-5b was reduced up to 8-fold when excess ATP was added to the actomyosin complex in the presence of varying concentrations of ADP. The dissociation reactions were monophasic and best described by single exponential fluorescence change to single exponentials and plotted against the mantADP concentration. Actin binding to Dd myosin-5b in the absence (●) and presence of ADP (■). The observed rate constants were plotted against the pyrene-actin concentration and second-order rate constants were obtained from linear fits to the data. All rate and equilibrium constants are summarized in Table 2.
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Table 2

Summary of the rate and equilibrium constants of the actomyosin interactions in the presence and absence of nucleotides

| Processive Dictyostelium Myosin-5b | Dd myosin-5b | Hs myosin-5b | Gg myosin-5a | Hs myosin-5c<sup>a</sup> | Dm myosin-5f<sup>b</sup> |
|-------------------------------------|--------------|--------------|--------------|----------------|------------------|
| **Nucleotide binding to myosin**    |              |              |              |                 |                  |
| ATP                                | 0.47 ± 0.02  | 0.31         | 1.6          | 2.5             | 1.31             |
| ADP                                | 5.5 ± 0.9    |              | 3.7          | 2.9             | 2.2              |
| mantADP                            | 0.92 ± 0.1   | 0.51         | 1.9          | 3.6             | 38.3             |
| **Nucleotide binding to actomyosin**|              |              |              |                 |                  |
| ATP                                | 0.19 ± 0.01  | 0.31         | 0.9          | 1.8/0.82        | 0.36             |
| ADP                                | 8 ± 1.3      | 870          | 16           | 17.4            | 15.8             |
| mantADP                            | 17.4 ± 1.7   | 11.7         | 12           | 17.7/15.6       | 150              |
| **Actin binding to myosin**         |              |              |              |                 |                  |
| k<sub>A</sub> (μM<sup>-1</sup>s<sup>-1</sup>) | 1.17 ± 0.08  | 73           | 1.1/0.66     | 2.5             |                  |
| k<sub>-A</sub> (s<sup>-1</sup>)       | 0.023 ± 0.001| 0.0036       | 0.011/0.019  | 0.04            |                  |
| k<sub>x</sub> (nm)                  | 20 ± 2       | 0.005        | 9.9/29       | 16              |                  |
| **Actin binding to myosin in the presence of ADP** |              |              |              |                 |                  |
| k<sub>AD</sub> (μM<sup>-1</sup>s<sup>-1</sup>) | 0.78 ± 0.06  | 4.2          | 0.88/1.17    | 2.3             |                  |
| k<sub>-AD</sub> (s<sup>-1</sup>)      | 0.03 ± 0.001 | 0.032        | 0.0099/0.051 | 0.43            |                  |
| k<sub>DA</sub> (nm)                 | 38.5 ± 5     | 7.6          | 11.3/44      | 190             |                  |
| **Duty ratio**                      | 0.74 ± 0.05  | 0.79         | 0.95         | 0.39/0.12       | 0.12             |
|                                   | 0.23 ± 0.05  |             |             |                 |                  |

<sup>a</sup> Experimental conditions: 25 mM MOPS, 100 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, pH 7.0.

<sup>b</sup> Watanabe et al. (21).

<sup>c</sup> De La Cruz et al. (19).

<sup>d</sup> Watanabe et al. (22).

<sup>e</sup> Takagi et al. (23).

<sup>f</sup> Tóth et al. (24).

<sup>g</sup> K<sub>AD</sub> (μM) = (k<sub>AD</sub>/k<sub>-AD</sub>)

<sup>h</sup> Calculated values according to Equation 2 at 5 mM free Mg<sup>2+</sup>-ion.

<sup>i</sup> Calculated values according to Equation 2 at 0.2 mM free Mg<sup>2+</sup>-ion concentrations.

The determined rate constants were plotted against the ADP concentration and the data were fitted to a hyperbola (Fig. 2B) yielding a dissociation equilibrium constant (K<sub>AD</sub>) of 8 μM. At high ADP concentrations the dissociation rate constant of the acto-Dd myosin-5b complex by 2 mM ADP decreased to 17.4 ± 1.9 s<sup>-1</sup>. Because ADP release from the A-M-D complex limits the rate of the ATP-induced dissociation, the rate of 17.4 s<sup>-1</sup> corresponds directly to the ADP dissociation rate from actomyosin (K<sub>-AD</sub>). F-actin has minimal effects on the affinity of ADP to Dd myosin-5b, although both binding and dissociation rates are affected by the presence of actin. In addition, the association constant of actin for Dd myosin-5b in the presence of ADP (K<sub>DA</sub>) was calculated as follows: K<sub>DA</sub> = K<sub>AD</sub>/k<sub>-AD</sub> × K<sub>A</sub>. The resulting affinity for actin in the presence of ADP (K<sub>DA</sub>) is 56 nm and comparable with the value calculated from of K<sub>DA</sub> = k<sub>-AD</sub>/k<sub>AD</sub> = 38.5 nm (Table 2).

**Effect of Free Mg<sup>2+</sup> Ions on ADP Binding Kinetics to Acto-Dd Myosin-5b**—We examined the kinetics of ADP binding to acto-Dd myosin-5b by mixing nucleotide-free acto-Dd myosin-5b with increasing concentrations of mantADP in the presence of 0.2, 1, 3, and 5 mM free Mg<sup>2+</sup>-ions. The resulting fluorescence increase followed single exponential functions at all conditions. The observed rate constants increased linearly with increasing ADP concentration (Fig. 3A). The second-order rate constant (k<sub>AD</sub>) determined from the slope of the linear fit to the data ranged from 4.0 ± 0.7 to 6.7 ± 0.4 μM<sup>-1</sup>s<sup>-1</sup>. In contrast, the ADP dissociation rates (k<sub>-AD</sub>) as obtained from the y intercepts of the straight lines decreased with increasing concentrations of free Mg<sup>2+</sup>-ions. The Mg<sup>2+</sup>-ion dependence of the ADP dissociation from the actomyosin complex was further confirmed by directly measuring the rate of ADP dissociation from acto-Dd myosin-5b using the fluorescent analogue mantADP. In the presence of 0.2, 1, 3, 4, and 5 mM free Mg<sup>2+</sup>-ions, the time courses for the observed fluorescence change after mixing acto-Dd myosin-5b-mantADP with 1 mM ADP follow single exponentials (Fig. 3B, inset). The apparent rate constant for mantADP release (k<sub>AD</sub>) dropped from 187 ± 25 s<sup>-1</sup> at 0.2 mM free Mg<sup>2+</sup> to 17.4 ± 1.9 s<sup>-1</sup> at 5 mM Mg<sup>2+</sup>. The observed inverse hyperbolic dependence of the rate of mantADP on the free Mg<sup>2+</sup> concentration is described by Equation 1,

\[
\text{k}_{-\text{AD}} = \frac{(k_{\text{max}} \cdot [\text{Mg}^{2+}]/K) + k_{\text{min}}}{([\text{Mg}^{2+}]/K) + 1} \tag{1}
\]

where k<sub>-AD</sub> is the observed rate constant, k<sub>max</sub> is the rate constant at 0 μM free Mg<sup>2+</sup>-ions, k<sub>min</sub> is the rate constant at saturating free Mg<sup>2+</sup>-ion concentrations, and K is the apparent inhibition constant for free Mg<sup>2+</sup>-ions, which is 0.44 ± 0.1 mM (Fig. 3B).

**Influence of Free Mg<sup>2+</sup> Ions on the Motility Activity of Dd Myosin-5b**—The motor activity of Dd myosin-5b was studied using the in vitro motility assay with a motor domain construct fused to an artificial lever-arm consisting of two α-actinioin repeats. The ATP concentration was kept constant at 4 mM and the free Mg<sup>2+</sup>-ion concentration was varied in the range from 0.01 to 20 mM. A maximum sliding velocity of 1.25 ± 0.12 μm/s...
and the data were fitted with a hyperbola. All rate and equilibrium constants were determined from the ADP inhibition of the ATP-induced dissociation of the actomyosin complex. Monophasic dissociation reactions were observed as the complex was dissociated with 1 mM ATP in the absence and presence of actomyosin complex. At 5 mM free Mg\(^{2+}\) ions the landing rate was observed at concentrations of free Mg\(^{2+}\)-ions between 10 and 50 \(\mu M\). Increasing concentrations of free Mg\(^{2+}\)-ions that are within the physiological range (0.1 to 1 mM) reduce the motile activity of \(Dd\) myosin-5b in a sigmoidal dependence up to 3-fold with an apparent inhibition constant for Mg\(^{2+}\)-ions \((K_i)\) of 0.43 ± 0.05 ms (Fig. 3C) that is well consistent with the \(K_i\) obtained from Fig. 3B (0.44 ± 0.1 mm).

To assay the effect of Mg\(^{2+}\)-ions on the ability of \(Dd\) myosin-5b to bind and move actin filaments at very low surface densities, we performed landing assays at free Mg\(^{2+}\)-ion concentrations corresponding to 5 and 0.28 mm. The number of landing events decreased as the surface density of myosin molecules decreased. At 5 mm free Mg\(^{2+}\)-ions the landing rate was best fit to the equation: 

\[
L(p) = \frac{L(1 - e^{-1/p(K_p)})}{1 + e^{-1/p(K_p)}},
\]

according to the model by Hancock and Howard (41) with \(p = 1.25 ± 0.2\) (Fig. 3D, solid circles) indicating that one \(Dd\) myosin-5b molecule is sufficient to bind and initiate movement. At 0.28 mm free Mg\(^{2+}\)-ions the number of landing events was drastically reduced and the data were best fit with \(p = 18 ± 2\) (Fig. 3D, open circles). At Mg\(^{2+}\)-ion concentrations <0.2 mm and myosin surface densities <3000 molecules/\(\mu m^2\) no landing events could be observed.

**Influence of Free Mg\(^{2+}\)-Ions on the Population of the Strong Actin Binding States and Duty Ratio of \(Dd\) Myosin-5b**—The influence of free Mg\(^{2+}\)-ions on the population of strong actin binding states was assayed by two different experimental approaches. First, the dissociation reaction of the actomyosin-5b complex by excess ATP was monitored at different free Mg\(^{2+}\)-concentrations (Fig. 4A). A complex of 2 \(\mu M\) \(Dd\) myosin-5b and 15 \(\mu M\) pyrene-actin was dissociated by 50 \(\mu M\) ATP at 5 and 0.2 mm free Mg\(^{2+}\)-ion concentrations. The amplitudes were normalized and set in relation to the maximum change in fluorescence of \(Dd\) myosin-5b binding to pyrene-actin. An initial fast fluorescence decrease describing the dissociation of the complex is followed by a second, slower phase of re-association. At 5 mm free Mg\(^{2+}\)-ion concentration the dissociation rate constant equals 4.2 s\(^{-1}\), which is ~3 times slower than the rate observed at 0.2 mm free Mg\(^{2+}\)-ions \((k_{obs} = 11.9 s^{-1})\). As this dissociation reaction describes the conformational transition of myosin from high to low actin affinity, the slow dissociation rate at 5 mm free Mg\(^{2+}\)-ions indicates that \(Dd\) myosin-5b stays for a longer fraction of time strongly bound to actin than at 0.2 mm free Mg\(^{2+}\)-ions.

To further evaluate whether \(Dd\) myosin-5b is a high duty ratio motor at high Mg\(^{2+}\)-concentrations and a low duty ratio motor at low Mg\(^{2+}\)-concentrations, we performed sequential mixing experiments. A pre-equilibrated mixture of 4 \(\mu M\) myosin-5b and 20 \(\mu M\) pyrene-actin was rapidly mixed with 400 \(\mu M\) ATP, aged for 50 ms to allow ATP-binding, hydrolysis, and population of the weakly bound states (A-M-D-P), and then quenched with 2 \(\mu M\) ADP to prevent ATP binding to myosin (Fig. 4B). The final concentrations of pyrene-actin and myosin-5b after double mixing are 5 and 1 \(\mu M\), respectively. The observed fluorescence quench is assumed as the transition from the high fluorescence weak binding states to the low fluorescence strong binding states (Scheme 1). At high concentrations of free Mg\(^{2+}\)-ions (5 mm, upper trace) the observed rate of the weak-to-strong transition is 7.0 ± 0.3 s\(^{-1}\) and the amplitude is 0.68. At 0.2 mm free Mg\(^{2+}\)-ions \(k_{obs}\) equals 1.5 ± 0.2 s\(^{-1}\) and the amplitude is decreased to 0.22 (Fig. 4B). Because both rate constants are similar to the steady-state ATPase rate (1.4 s\(^{-1}\)) that was obtained at 5 \(\mu M\) actin in the presence of 0.2 and 5 mm free Mg\(^{2+}\)-ions, we conclude that ATP turnover is limited by the weak-to-strong transition in both cases. Thus, free Mg\(^{2+}\)-ions have no influence on the actin activated steady-state ATPase rates. However, the differences in the amplitude of the pyrene-actin fluorescence at 0.2 and 5 mm free Mg\(^{2+}\)-ions indicate that 68 and 22% of the \(Dd\) myosin-5b molecules, respectively, are strongly bound to actin. These values provide direct estimates for the duty ratio (19).

**Free Mg\(^{2+}\)-Ion Distribution in Dictyostelium Cells**—To further elucidate the physiological relevance of the observed functional changes upon variation of the free Mg\(^{2+}\)-ion concentra-
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**FIGURE 3. Mg\(^{2+}\) dependence of ADP binding kinetics and motor activity.** A, effect of free Mg\(^{2+}\)-ions on mantADP binding to acto-Dd myosin-5b at 0.2 mM (○), 1 mM (△), 3 mM (□), and 5 mM (■) concentrations of free Mg\(^{2+}\)-ions. The slope of each plot defines an apparent second-order rate constant (k\(_{AD}\)) for ADP binding to myosin and the y intercept defines a dissociation rate constant (k\(_{AD}\)). B, Mg\(^{2+}\) dependence of mantADP dissociation from acto-Dd myosin-5b. The displacement of mantADP from the ternary mantADP-acto-Dd myosin-5b complex by excess unlabeled ADP at concentrations of free Mg\(^{2+}\)-ions from 0.2 to 5 mM was monitored from the resulting decrease of mant-fluorescence (inset). The data traces represent the averages of at least eight independent transients. The transients follow single exponentials and the observed dissociation rate constants (k\(_{AD}\)) decrease in an inverse hyperbolic manner with increasing free Mg\(^{2+}\)-concentrations. Excess free Mg\(^{2+}\)-concentrations lead to a more than 10-fold decrease in the mantADP dissociation rate constant.

C, dependence of Dd myosin-5b motor activity on free Mg\(^{2+}\)-ions. Uncertainties represent mean ± S.D. of at least three independent experiments. D, landing rate as a function of Dd myosin-5b surface density in the presence of 5 mM (filled circles) and 0.28 mM (open circles) free Mg\(^{2+}\)-ions. The data were fit to equation: L(t) = Z(1 − e\(^{-kt}\)), as described in Ref. 41, yielding values of n = 1.25 for 5 mM free Mg\(^{2+}\)-ions (solid line) and n = 18 for 0.28 mM free Mg\(^{2+}\)-ions (dashed line), respectively. Each data point and the corresponding standard deviations were derived from three independent measurements from one or two flow cells.

**DISCUSSION**

*Dictyostelium* myosin-5b is a dimeric motor that consists of two heavy chains, each with a molecular mass of 258 kDa and six associated light chains. *Dictyostelium* myosin-5b is assumed to localize at the contractile vacuole (28), a specialized organelle for the regulation of osmotic pressure (44). Recent phylogenetic analyses of more than 1700 myosins from various species places *Dictyostelium* myosin-5b and the closely related smaller isozyme *Dd* myosin-5a (previously referred to as Myo H) among the class 5 myosins (45, 46). Notable kinetic features of *Dd* myosin-5b emphasizing its close relation to processive class 5 myosins are summarized in Tables 1 and 2 and include slow rates of ADP release (k\(_{AD}\)), ATP binding to actomyosin (k\(_{AD}\)), and ATP-induced actin dissociation (k\(_{AD}\)). Additionally, ATP hydrolysis is fast (k\(_{AD}\)), ADP has only a minor effect on actin binding and dissociation (k\(_{AD}\)), and coupling between actin and nucleotide binding (K\(_{AD}\)/K\(_{AD}\)) is low. The simultaneous occurrence of these kinetic properties is indicative for *Dd* myosin-5b being a high duty ratio motor that populates predominantly the strong actin binding states A-M and A-M-ADP (Scheme 1). The high duty ratio derives primarily from the slow rate of ADP release, which becomes rate-limiting for steady state turnover in the presence of actin. A slow rate of ATP-binding to actomyosin and a fast rate of ATP hydrolysis (>300 s\(^{-1}\)) contribute further to a predominant population of strong binding states. However, particular steps in the ATPase cycle of *Dd* myosin-5b do critically depend on the concentration of free Mg\(^{2+}\)-ions. We have reported earlier that changes in the physiological concentration of free Mg\(^{2+}\)-ions modulate the kinetic properties of *Dd* myosin-1E (38) and *Dd* myosin-1D (47). High free Mg\(^{2+}\)-concentrations reduce the motile activity of these myosin motors by inhibiting the rate of ADP dissociation from actomyosin. As a consequence, high concentrations of free Mg\(^{2+}\)-ions stabilize the tension-bearing acto-myosin-ADP state and shift the actomyosin system from the production of rapid movement toward the generation of tension. In an independent study with vertebrate myosin-5a Sweeney and co-workers (48) reported that free Mg\(^{2+}\)-ions have an influence on the rate and order of product release. In accordance to these studies, we...
have observed that high free Mg\(^{2+}\)-ion concentrations lead to a reduction in the coupling ratio of actin and ADP binding of *Dd* myosin-5b ($K_{AD}/K_D \approx 1$). Under these conditions *Dd* myosin-5b is able to bind both F-actin and ADP with high affinity. However, in the presence of low free Mg\(^{2+}\)-ion concentrations, $K_{AD}$ is 5-fold increased (27.9 M), $K_D$ is almost unaffected, and the coupling ratio ($K_{AD}/K_D$) is $\sim 5$-fold increased. Our *in vitro* motility experiments show that Mg\(^{2+}\)-dependent alterations in this order of magnitude directly affect the motile activity of the motor. At $\sim 50$ M free Mg\(^{2+}\)-ions *Dd* myosin-5b displays its highest sliding velocity, whereas free Mg\(^{2+}\)-ion concentration $> 1$ mM reduce the velocity up to 3-fold. This behavior is due to the reduced ADP release rate from actomyosin, which in turn results in an increased accumulation of the strong binding intermediate state A-M-ADP. From this experiment we conclude that high physiological concentrations of free Mg\(^{2+}\) can extend the time the motor spends in strongly bound states up to 3-fold. We investigated this behavior by determining the duty ratio of the motor at low and high free Mg\(^{2+}\) concentrations with the aid of the Equation 2, where $T_{strong}$ defines the time the motor spends in the strong actin binding states and $T_{total}$ the overall ATPase cycle time.

$$\text{Duty ratio} = \frac{(1/k_{AD}) + (1/k_{+2})}{(1/k_{cat})} = \frac{T_{strong}}{T_{total}} \quad \text{(Eq. 2)}$$

Using the parameters $k_{AD} = 21.6$ s$^{-1}$, $k_{+2} = 75$ s$^{-1}$, and $k_{cat} = 12.4$ s$^{-1}$ that were obtained at 5 mM free Mg\(^{2+}\)-ions according to Tables 1 and 2, the calculated duty ratio of *Dd* myosin-5b equals 0.74. This value is in good agreement with the experimentally determined duty ratio of 0.68 that was obtained under the same conditions at 5 M F-actin concentrations (Fig. 4B). Because the duty ratio depends on the actin concentration, it is expected that higher actin concentrations would lead to a further increase of the duty ratio, thus approaching the calculated value of 0.74, which is representative for high actin concentrations. In contrast, at low concentrations of free Mg\(^{2+}\)-ions (0.2 mM), the rate of ADP release from acto-*Dd* myosin-5b increases $\sim 10$-fold (from 21.6 to 187 s$^{-1}$). ATP binding becomes 3-fold faster (Fig. 4A), and the fraction of strongly bound states decreases more than 3-fold (Fig. 4B). The calculated and experimentally determined duty ratio is identical under these conditions (duty ratio $= 0.23$). The modulation of velocity and duty ratio suggests that *Dd* myosin-5b can switch between working either as a stepping motor or as a motor that is adapted for tension bearing. The results from the landing assays confirm this hypothesis and indicate that *Dd* myosin-5b is a processive motor in the higher physiological range of free Mg\(^{2+}\)-ion concentrations and a non-processive in the lower range. We conclude that this kind of conditional adaptation of the mechanoenzymatic mechanism is important in cellular processes that require a cyclical switching between fast contractile activity and slow tension bearing. In agreement with this assumption, our results show that *Dd* myosin-5b localizes at the contractile vacuole (see Fig. 5 and supplemental Movie 1), where it appears to be actively involved in the fast contraction phase and the subsequent phase when expansion of the vacuole volume is suppressed.

The concentration of free Mg\(^{2+}\)-ions inside the cell varies in the range from 0.1 to 1 mM (42). The use of a new generation of Mg\(^{2+}\)-sensitive fluorescent dyes allows the direct observation
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of these changes with greater special and temporal resolution. Here, we have shown that similar changes as observed for HL60, HC11, and PC12 cells (49, 50) do occur in Dictyostelium (see Fig. 5B and supplemental Movie 2). The observed fluctuations take place in the millisecond range and thus within a time domain where the consequences for myosin motor activity are of physiological relevance.

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