A Unique ATP Hydrolysis Mechanism of Single-headed Processive Myosin, Myosin IX*

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Recent studies have revealed that myosin IX is a single-headed processive myosin, yet it is unclear how myosin IX can achieve the processive movement. Here we studied the mechanism of ATP hydrolysis cycle of actomyosin IXb. We found that myosin IXb has a rate-limiting ATP hydrolysis step unlike other known myosins, thus populating the prehydrolysis intermediate (M-ATP). M-ATP has a high affinity for actin, and, unlike other myosins, the dissociation of M-ATP from actin was extremely slow, thus preventing myosin from dissociating away from actin. The ADP dissociation step was 10-fold faster than the overall ATP hydrolysis cycle rate and thus not rate-limiting. We propose the following model for single-headed processive myosin. Upon the formation of the M-ATP intermediate, the tight binding of actomyosin IX at the interface is weakened. However, the head is kept in close proximity to actin due to the tethering role of loop 2/large unique insertion of myosin IX. There is enough freedom for the myosin head to find the next location of the binding site along with the actin filament before complete dissociation from the filament. After ATP hydrolysis, Pi is quickly released to form a strong actin binding form, and a power stroke takes place.

Among the myosin superfamily members, class IX myosin is quite unique in several aspects. One of the unique features is that the tail region contains a GTPase-activating protein domain for the small GTP-binding protein, Rho (1, 2). Thus, myosin IX may be a motor protein carrying its Rho-GTPase-activating protein tail to required sites to down-regulate Rho-dependent signaling. Quite recently, it was reported that myosin IXb binds to BIG1, a guanine nucleotide exchange factor for ADP-ribosylation factor (Arf1) and the binding of BIG1 to myosin IXb competes with RhoA binding, thus regulating the Rho-GTPase-activating protein activity of myosin IXb (3). These findings raise an idea that myosin IX delivers the G-protein signaling molecules to the cellular compartment, where G-protein regulation takes place. Supporting this view, it is shown that myosin IXb moves processively on actin filaments (4, 5). Interestingly, it is found that myosin IXb moves toward the minus end of the actin filament (5). Subsequently, it was reported that the full-length myosin IXb moves toward the plus end of actin filament (6), and because the construct of myosin IXb used by Inoue et al. (5) does not have the tail domain, it was proposed that the tail domain may be important for the determination of the directionality of myosin IXb.

A critical issue is that myosin IXb is a single-headed myosin yet moves processively on actin filaments. It has been proposed that the two-headed processive myosin, such as myosin V, moves on actin filaments with a hand-over-hand mechanism to travel a long distance (7, 8). Two critical features are required for this mechanism. One is the high duty ratio, in which each myosin head spends a majority of the ATP hydrolysis cycle time (more than 50%) with the strong actin-binding form. During the ATPase cycle, myosin populates either the weak-binding state or strong binding state. The rate-limiting step of a nonprocessive motor, such as myosin II, is Pi release (k<sub>i</sub>Pi); thus, nonprocessive myosins spend a large fraction in the weak binding during the ATPase cycle. Therefore, those myosins can work together in an asynchronous ensemble with high speed. On the other hand, the rate-limiting step for myosin V is ADP release (k<sub>i</sub>ADP); thus, the processive myosins spend the strong binding state in a large fraction during the ATPase cycle. The other is the coordinated binding of the two heads to actin. If the fraction of strong binding state for a single head of myosin V is greater than 0.5, double-headed processive myosin can move on actin filament continuously through a coordinated interaction of two motor domains with the actin filament. However, this model cannot account for the processive movement of a single-headed myosin IX. Of interest is how single-headed myosin IX moves processively on actin filament.

Here we studied the ATP hydrolysis mechanism of actomyosin IX to clarify such unique features of myosin IX, a single-headed processive myosin, by analyzing the ATP hydrolysis mechanism that is closely coupled with the cross-bridge cycling of myosin.

**EXPERIMENTAL PROCEDURES**

Reagents and Proteins—2'-Deoxy-mant-ATP (dmantATP) was kindly provided by Dr. Howard D. White (Eastern Virginia Medical School). Rabbit skeletal muscle actin was purified according to Spudich and Watt (9). Recombinant human calmodulin was cloned from human testis total RNA (Clontech). The cDNA was synthesized by reverse transcription with random oligonucleotides and subcloned into pFastbac vector for expression in insect Sf9 cells. The CaM cDNA was also introduced to PET30 vector for expression in E. coli and purified as described (10).

Preparation of Recombinant Myosin IXb—The myosin IXb construct used (M9bIQ4) was prepared previously (5). The construct contains nucleotides 1–3889, encoding residues 1–1296 of human myosin IXb (Fig. 1A). The recombinant myosin IXb was expressed as described (5). Typically, 0.3 mg of protein is obtained from 300 ml of culture.

**Gel Electrophoresis**—SDS-PAGE was carried out on a 5–20% polyacrylamide gel using the discontinuous buffer system of Laemmli (11). **Steady-state ATPase Assay**—The ATPase assays were performed at 25°C in 30 mM HEPES, pH 7.5, 30 mM KCl, 1 mM EGTA, 2 mM MgCl<sub>2</sub>, 1 mM DTT, and 10 μM CaM (buffer A). Liberated 32P was measured as described previously (12).

**Actin Binding Assay**—Various concentrations of actin (1–20 μl) were mixed with 0.3–0.5 μl M9bIQ4 in buffer A and allowed to stand

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2 The abbreviations used are: dmantATP, 2'-deoxy-mant-ATP; mant, methylanthraniloyl; CaM, calmodulin; M9bIQ4, myosin IXb truncated at the end of IQ motif; VI, vanadate.
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for 5 min at room temperature. 2 mM ATP was added and incubated for 5 min at room temperature. For the binding assay of the M-ADP-P state, 0.1 mM ADP and 1 mM Vi were incubated with M9bIQ4 for 1 h on ice, and then various concentrations of actin were incubated with the ternary complex of M9bIQ4-ADP-Vi for 5 min at room temperature. The samples were centrifuged in the Beckman Optima TLX Ultracentrifuge at 300,000 × g for 5 min. Equal proportions of supernatant and dissolved pellet were run on SDS-polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue R-250. The band intensities were quantified using the ImageJ software to determine the percentage of M9bIQ4 bound to pelleted actin.

To determine the bound M9bIQ4 containing ADP-Vi, the amount of [3H]ADP trapped in acto-M9bIQ4 was measured. M9bIQ4 was incubated with [3H]ADP in the presence and absence of 1 mM ADP for 1 h on ice, and then 20 μM actin and 2 mM cold ADP were added to the mixture. If [3H]ADP-Vi is not stably trapped in the active site of M9bIQ4, [3H]ADP is replaced by nonradioactive ADP. After incubation for 5 min at room temperature, the samples were ultracentrifuged. The pellet was washed with the buffer containing 1 mM ADP, and the amount of [3H]ADP in dissolved pellet was counted by a scintillation counter. The concentration of bound M9bIQ4 in dissolved pellet was also determined by densitometry of an SDS-polyacrylamide gel, and the percentage of trapped [3H]ADP in the M9bIQ4 was calculated.

Photoaffinity Labeling of Myosin IXb with ATP—Photoaffinity labeling was performed as described by Maruta and Korn (13), with some modification. Myosin IXb was mixed with 0.1 mM [γ-32P]ATP or [γ-32P]ATP in buffer A and 0.5 mg/ml bovine serum albumin. After irradiation for 2 min with UV light at 254 nm, the proteins were precipitated by the addition of 5% trichloroacetic acid containing 1% sodium pyrophosphate. The pellets were subjected to SDS-PAGE. Incorporation of 32P into myosin heavy chain was analyzed by a phosphor imager (Fuji Film FLA-5000).

Kinetic Experiments—All transient kinetic experiments were done in buffer A at 25 °C using Kin-Tek SF-2001 stopped flow apparatus. The concentration of M9bIQ4 after mixing was 0.2–0.6 μM, and actin was added at 1.2 times the M9bIQ4 concentration. Fluorescence change of dmantATP was measured by fluorescence energy transfer by exciting at 280 nm, and emission was detected at 400 nm with a long pass filter (Oriel). For 90° light scattering, the excitation beam was passed through a 360-nm interference filter.

Quenched flow measurements were performed with a Kin-Tek RQF-3 apparatus (KinTek Corp.). Samples of M9bIQ4 or acto-M9bIQ4 were mixed with an equal volume of [32P]ATP in buffer A. After aging in the delay line, reactions were stopped by mixing with a solution containing 0.3 M perchloric acid, and the liberated 32P was measured.

All of the transients shown are the average of 3–6 independent mixings. Single exponential data were fit to the equation \( I(t) = c + I_0 \exp(-k_{obs}t) \), and two exponential data were fit to \( I(t) = c + I_1 \exp(-k_{obs1}t) + I_2 \exp(-k_{obs2}t) \), where \( I(t) \) is the fluorescent signal at time \( t \), \( c \) is constant, and \( I_1, I_2, \) and \( k_{obs1}, k_{obs2} \) are the amplitude coefficients of reactions with rate constant \( k_{obs1} \) and \( k_{obs2} \), respectively. Kinetic modeling and simulation were performed using STELLA software version 8.1.1 (Isee Systems).

RESULTS

We expressed and purified the M9bIQ4 construct (Fig. 1A) that contained the entire head domain and the light chain binding neck domain, because this shows the processive movement on actin filament, and one can avoid the potential complexity arising from the presence of the tail domain containing the Rho-binding GTPase-activating protein domain. The purified construct was composed of a heavy chain band and calmodulin and free from 200-kDa S9 conventional myosin and 43-kDa actin (Fig. 1B). The number of calmodulin associated with M9bIQ4 was determined by densitometry of an SDS-polyacrylamide gel and estimated to be as follows: M9bIQ4 heavy chain/calmodulin = 1:3.93 ± 0.06.

Steady-state ATPase Activity of M9bIQ4—Interestingly, the ATPase activity is not significantly activated by actin (Table 1, Fig. 2). The steady-state ATPase activity at saturating ATP concentration is 0.22 s⁻¹ with a \( K_{ATP} \) of 7.95 μM in the absence of actin and 0.29 s⁻¹ with a \( K_{ATP} \) of 6.30 μM in the presence of actin.

![Figure 1](image1.png)

**FIGURE 1. Myosin IX construct.** A, schematic diagram of expressed truncated human myosin IXb (M9bIQ4). The molecule is monomeric. B, purified M9bIQ4. CaM undergoes its characteristic Ca²⁺-dependent shift in mobility (lane 2, EGTA; lane 3, Ca²⁺).

**TABLE 1**

| Actin (μM) | \( V_{max} \) | \( K_{ATP} \) | \( K_{actin} \) |
|-----------|--------------|--------------|--------------|
| 0         | 0.22 (0.012) | 7.95 (0.77)  | 2.3 (2.1)    |
| 20        | 0.29 (0.015) | 6.30 (0.62)  | 2.3 (2.1)    |

![Figure 2](image2.png)

**FIGURE 2. The steady-state ATPase activity as a function of actin concentration.** The ATPase activity of M9bIQ4 (0.1–0.2 μM) was measured as a function of actin concentration in the presence of 0.3 mM ATP. Solid lines, calculated based on the equation, \( V = V_{max}\text{actin}/(K_{actin} + \text{[actin]}) + V_0 \). According to the analysis, the basal ATPase activity (V₀) is obtained for 0.22 s⁻¹. The maximum activation by actin (Vₘₐₓ) is 0.07 s⁻¹. The maximum ATPase activity at saturating actin concentration (Vₘₐₓ + V₀) is 0.29 s⁻¹ with a \( K_{actin} \) of 2.3 μM. The error bars indicate S.D. for \( n = 3 \) from three independent preparations.
As demonstrated for other characterized processive myosins, myosin V and myosin VI, ATPase activity of processive myosins is inhibited by ADP because of slow dissociation of ADP from the strong actin binding form of myosin (14, 15). In contrast to myosin V and myosin VI, the ATPase activity of M9bIQ4 was not changed in the absence and presence of the ATP regeneration system (Fig. 3A), suggesting that the ATPase of M9bIQ4 is not inhibited by ADP. To further confirm this notion, the ATPase activity of M9bIQ4 was measured as a function of ADP. The ATPase activity of M9bIQ4 was not inhibited by ADP. To further confirm this notion, the ATPase activity of M9bIQ4 was measured as a function of ADP (Fig. 3B). The experiment was done with low ATP concentration (25 μM), because ADP only moderately inhibited the ATPase activity. A K_{ADP} value of 16 μM was obtained. In the presence of 0.5 mM ATP, the inhibition of the ATPase activity of M9bIQ4 is not observed in the presence of the ATP regeneration system (Fig. 3).

**SCHEME 1.** ATP hydrolysis cycle of actomyosin.

**TABLE 2**  
Kinetische and equilibrium constants for actin-activated ATPase of M9bIQ4

| Process       | Value       | Derivation |
|---------------|-------------|------------|
| ATP binding   | K_{ATP} (μM<sup>-1</sup> s<sup>-1</sup>) | MnATP      |
|               | k<sub>-1</sub> (s<sup>-1</sup>) | 1.08 (0.21) Mn      |
|               | k<sub>1</sub> (μM<sup>-1</sup> s<sup>-1</sup>) | 2.22 (0.71) Mn      |
|               | 1/K<sub>v</sub> (μM) | 2.06 Calculation |
|               | K<sub>1</sub>/K<sub>2</sub> (μM<sup>-1</sup> s<sup>-1</sup>) | 1.07 (0.10) Mn      |
|               | k<sub>-2</sub> (s<sup>-1</sup>) | 3.43 (0.33) Mn      |
|               | 1/K<sub>1</sub> (μM) | 3.21 Calculation |
| Hydrolysis    | k<sub>v</sub> (s<sup>-1</sup>) | 0.21–0.25 Simulation |
|               | k<sub>1</sub>v (s<sup>-1</sup>) | 0.4–0.45 Simulation |
| ADP release   | K<sub>ADP</sub> (μM) | 3.34 (0.28) Light scattering |
|               | k<sub>-2</sub> (s<sup>-1</sup>) | 0.21 Calculation |
|               | k<sub>1</sub> (μM<sup>-1</sup> s<sup>-1</sup>) | 16.0 (1.3) Steady state |

**FIGURE 3.** Moderate inhibition of the steady state ATPase activity of M9bIQ4 by ADP. A, time course of the steady-state ATPase activity of M9bIQ4 (0.35 μM) in the presence of actin with or without the ATP-regenerating system. The ATPase activity was measured in the presence of 25 μM ATP, 10 μM actin, and various concentrations (0–1 mM) of ADP. The data were fit to the equation, v = V<sub>max</sub>[ATP]/(K<sub>vATP</sub> + [ADP]/K<sub>ADP</sub> + [ATP]), where [ATP] is 25 μM and K<sub>vATP</sub> is 6.3 μM. According to the analysis, K<sub>ADP</sub> was obtained for 16 μM. The error bars indicate S.D. for n = 3 from three independent preparations.

**FIGURE 4.** Kinetics of dmantATP binding to M9bIQ4 and acto-M9bIQ4. Rates of dmantATP binding to M9bIQ4 (open circles) and acto-M9bIQ4 (closed circles) as a function of nucleotide concentration are shown. The observed rates (k<sub>obs</sub>) were obtained by fitting the fluorescence data at each nucleotide concentration to a single exponential. The apparent second order rate constants for dmantATP binding to M9bIQ4 and acto-M9bIQ4 are 1.08 and 1.07 μM<sup>-1</sup> s<sup>-1</sup>, respectively. Dissociation rates of ATP determined by y intercept are 2.22 s<sup>-1</sup> in the absence of actin and 3.43 s<sup>-1</sup> in the presence of actin. The error bars indicate S.D. for n = 4 from three independent preparations. The inset shows a dmantATP fluorescence transient obtained by mixing 0.25 μM M9bIQ4 or acto-M9bIQ4 with 3 μM dmantATP. The rates are 6.0 s<sup>-1</sup> in the absence of actin and 6.78 s<sup>-1</sup> in the presence of actin.
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![Fig. 5. ATP hydrolysis step of M9bIQ4. A, kinetics of ATP hydrolysis of M9bIQ4 and acto-M9bIQ4. Single turnover quench-flow measurements of hydrolysis are done upon mixing 0.85 mM M9bIQ4 (open circles) or acto-M9bIQ4 (closed circles) with 0.2 mM [γ-32P]ATP. The solid lines are the best fits to a single exponential. The apparent rate constants are 0.06 s⁻¹ in the absence of actin and 0.08 s⁻¹ in the presence of actin. The error bars indicate S.D. for n = 3 from two independent preparations. B, simulation for hydrolysis rate determined by the apparent rate of hydrolysis. Rate constants of hydrolysis (k₁, k₋₁) to fulfill experimentally determined kinetic constants (k_app = 0.06 s⁻¹ and k_app = 0.08 s⁻¹) are simulated using STELLA software. The experimentally determined kinetic constants (Table 2) were fed into a kinetic model according to Scheme 1. Parameters used were k₁, k₋₁ = 1.08 μM⁻¹ s⁻¹, k₋₂ = 2.22 s⁻¹, k₁k₋₂, = 1.07 μM⁻¹ s⁻¹, k₋₁ = 3.43 s⁻¹. M9bIQ4 or acto-M9bIQ4 = 0.85 μM, and [ATP] = 0.2 μM. Open circles, in the absence of actin. Closed circles, in the presence of actin. k₋₁ and k₋₂ were calculated to be 0.24 and 0.45 s⁻¹, respectively.

![Fig. 6. Photoaffinity labeling of myosin IXb with ATP. Predominant intermediate during ATPase is determined by photoaffinity labeling of myosin IXb using [α-32P]ATP or [γ-32P]ATP. Before (−UV) and after (+ UV) irradiation, samples were subjected to SDS-PAGE. Then the incorporation of [32P] into myosin heavy chain was analyzed by a phosphor imager. Smooth muscle myosin heavy meromyosin (Sm HMM) was used for control. Top panels, Coomassie Brilliant Blue (CBB) staining of the myosin heavy chain; lower panels, phosphor imager analysis of the myosin heavy chain. α and γ represent labeling with [α-32P]ATP or [γ-32P]ATP.]

The result is due to the lower affinity of ADP compared with that of ATP.

**ATP Binding**—The data of steady-state ATPase activity show that single-headed myosin IX has lower affinity for ADP, unlike other characterized prossive myosins, suggesting that the mechanism of propassive movement for myosin IXb would be different from previously reported two-headed prossive myosins, whose ADP release step is slow, and is the rate-determining step for the entire hydrolysis cycle (16, 17).

To clarify the mechanism of the propassive movement of single-headed myosin IX, we determined each kinetic step of the ATP hydrolysis cycle that is correlated to the cross-bridge cycle (Scheme 1). The fluorescent nucleotide dmantATP was used as a probe to measure the rate of nucleotide binding. A fluorescence enhancement of dmantATP upon binding to M9bIQ4 was best fit to a single exponential (Fig. 4, inset), and the rates are linearly related to the dmantATP concentration to yield the second order rate constants for dmantATP binding of K₁k₋₂, = 1.08 μM⁻¹ s⁻¹ and K₁k₋₂, = 1.07 μM⁻¹ s⁻¹, to M9bIQ4 and acto-M9bIQ4, respectively (Fig. 4). The y intercept indicates a dissociation rate of dmantATP (k₋₁, k₋₂) for M9bIQ4 and acto-M9bIQ4 to be 2.22 and 3.43 s⁻¹, respectively (Table 2). Based upon these parameters, the rate of ATP binding at physiological ATP concentration (>2 mM) (18) is >2000 s⁻¹, which is at least 6000-fold faster than the cycle rate if the rate constant is linearly increased with ATP. We found no sign of curvature within the ATP concentration tested, and the observed rate constant at 5 μM ATP far exceeded the ATPase cycle rate.

**ATP Hydrolysis**—All previously characterized myosins rapidly hydrolyze ATP to form the M·ADP·P complex (Pi burst), which is followed by slow product release. It has been thought that the formation of the myosin-ADP-P complex is necessary for normal motor function for myosin (19–21). The rate of hydrolysis was measured directory by using a quench-flow apparatus.

We employed a single turnover experiment, in which all given ATP binds to the myosin active site and is hydrolyzed. The kinetics for single turnover of all myosin so far known showed two exponentials of which the first rapid phase is due to rapid binding and hydrolysis of ATP, followed by the second slow phase of product dissociation. Surprisingly, unlike other myosins, the kinetics of ATP hydrolysis was very slow and best fit to a single exponential in both the presence and absence of actin (Fig. 5A). The result suggests that M9bIQ4 does not form M·ADP·P rapidly, and the rate of product off is faster than that of hydrolysis. The apparent hydrolysis rates of single turnover of ATP hydrolysis are 0.06 and 0.08 s⁻¹ in the absence and presence of actin, respectively. The values were slower than the steady state cycle rate because of the presence of significant reverse reaction of the ATP binding step unlike well known myosin I, II, and V. Fig. 5B shows the simulation to obtain k₋₁, and k₋₂ values from kobs with obtained rate constants of the ATP binding step. At given M9bIQ4 and ATP concentration, k₋₁ and k₋₂ values

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**TABLE 3**

| ATP      | kobs (s⁻¹) | Simulated k₋₁, to obtain kobs |
|----------|------------|-----------------------------|
| Without actin |             |                             |
| 0.5 μM M9bIQ4 | 0.1         | 0.038                       | 0.21 |
| 0.5 μM M9bIQ4 | 0.3         | 0.040                       | 0.23 |
| 0.43 μM M9bIQ4 | 0.2         | 0.039                       | 0.25 |
| 0.85 μM M9bIQ4 | 0.2         | 0.062                       | 0.25 |

| With actin |             |                             |
| 0.43 μM M9bIQ4 | 0.2         | 0.041                       | 0.40 |
| 0.85 μM M9bIQ4 | 0.2         | 0.079                       | 0.46 |

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The presence of 1 mM ADP, which is consistent with the above experiment. The result is due to the lower affinity of ADP compared with that of ATP.

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were estimated to be 0.24 and 0.45 s\(^{-1}\) in the absence and presence of actin, respectively. Table 3 summarizes the hydrolysis rate obtained from \(k_{\text{obs}}\) in different M9bIQ4 and ATP concentration. These rates agree well with those of steady-state ATP hydrolysis cycle rates, suggesting that the ATP hydrolysis is the rate-limiting for the M9bIQ4 ATPase reaction.

It is known that the intrinsic tryptophan fluorescence of myosin is enhanced upon nucleotide binding and is further enhanced upon the hydrolysis of ATP. It is thought that a conserved tryptophan residue (16, 22–31) located on the rigid relay loop is the largest contributor to the observed intrinsic fluorescence enhancement associated with nucleotide binding and hydrolysis. Whereas myosin IX contains this conserved Trp, ATP-induced change in Trp fluorescence was not observed (not shown).

Predominant Intermediate during ATPase Cycle—If the ATP hydrolysis step is the rate-limiting step for the entire cycle, the predominant reaction intermediate should be M-ATP rather than M-ADP-P. To support this notion, we determined the dominant intermediate by performing photoaffinity labeling of M9b with \(^{32}\text{P}\)-labeled ATP. Myosins were UV-irradiated with \([\alpha-^{32}\text{P}]\text{ATP}\) or \([\gamma-^{32}\text{P}]\text{ATP}\), and the incorporation of \(^{32}\text{P}\) into myosin heavy chain was analyzed by a phosphor imager. For conventional myosin (smooth muscle heavy meromyosin), the radioactivity was detected only when myosin was irradiated with \([\alpha-^{32}\text{P}]\text{ATP}\), but not with \([\gamma-^{32}\text{P}]\text{ATP}\) (Fig. 6). Since smooth muscle heavy meromyos-
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Actin Binding and ADP Off—It is known for reported myosins that the myosin head is rapidly dissociated from actin upon the addition of ATP; however, we found that the majority of myosin IX co-sedimented with actin even in the presence of ATP. This suggested that the actin binding properties of myosin IX are different from other myosin so far reported. It should be noted that M9bIQ4 was not precipitated in the absence of actin.

We further examined the unique actin binding properties of myosin IX. Light scattering was used to determine the rate of actin binding of M9bIQ4. The rate constants of the binding of M9bIQ4 and M9bIQ4-ADP to actin were linearly related to actin concentration (Fig. 7). The y intercept of Fig. 7 is very close to the original point in both presence and absence of ADP, suggesting that the off rate is very small. The apparent second order rate constant for M9bIQ4 binding to actin was 5.2 μM⁻¹ s⁻¹ in the absence of ADP (kₘₐₓ) and 5.4 μM⁻¹ s⁻¹ in the presence of ADP (kₘₐₓ ADP).

The rate of dissociation of M9bIQ4 and M9bIQ4-ADP from actin was also measured by light scattering. The kinetics of ATP-induced dissociation of acto-M9bIQ4 was slow and showed hyperbolic dependence on ATP concentration (Fig. 8). The maximum rate of dissociation was kₐₐ₈ + kₐ₈[actin] = 13.0 s⁻¹. This is extremely slow compared with other characterized myosins that the rate of dissociation is typically >500 s⁻¹.

The dissociation rate was significantly decreased in the presence of ADP, and this is because the dissociation of M9bIQ4 from actin should be limited by the rate of ADP release (kₐ₈) in the presence of excess ATP. Thus, the dissociation rate of ADP from acto-M9bIQ4 of kₐ₈ = 3.34 s⁻¹ was obtained, which is 10-fold faster than the entire ATP hydrolysis cycle rate. The rate of ADP off was also measured by monitoring the change in the fluorescence of bound dmantADP after the addition of excess ATP, and the result confirmed the light-scattering experiment (not shown). The result suggests that ADP release is not the rate-determining step for M9bIQ4. Since the affinity of ADP to acto-M9bIQ4 (Kₐ₈) is 16 μM (Fig. 3B), the association rate constant for ADP to acto-M9bIQ4 was calculated to be 0.21 μM⁻¹ s⁻¹. For other processive myosins, ADP release is rate-limiting, resulting in the formation of predominant intermediate at M-ADP state, which is strongly bound to actin (16, 17). The result suggests that the mechanism of processive movement of myosin IX is quite different from those of two-headed processive myosin.

We determined the binding affinity of M9bIQ4 to actin by co-sedimentation assay in the presence of ATP (Fig. 9A). The data points were fit to hyperbola, and the affinity of M9bIQ4 for actin in the presence of ATP was Kₐ₈ = 2.33 μM. Since the predominant intermediate is M-ADP, the Kₐ₈ value is considered as Kₐ₈ (equal to kₐ₈/kₐ₈). Since kₐ₈ + kₐ₈[actin] is equal to 13.08 s⁻¹, where [actin] = 0.6 μM, the values of kₐ₈ and kₐ₈ were calculated to be 5.45 μM⁻¹ s⁻¹ and 12.7 s⁻¹, respectively. The results indicate that M-ADP state for myosin IX has much higher binding affinity than other myosins, in which the M-ADP state is a weak actin binding state (32–34), including processive myosin VI (35). Consistent with this result, we found that the amplitude of the ATP induced change in the light scattering of acto-M9bIQ4 decreases with actin concentration. The extent of the change at 2 μM actin was approximately half of the maximum change in the light scattering estimated from the binding of M9bIQ4 to actin (not shown).

It is known for other myosins that M-ADP-P state is also a weak actin binding state. Because M-ADP-P is not the predominant intermediate for myosin IX, we used Vi to trap ADP into the active site. M9bIQ4 with excess [³H]ADP was incubated with or without vanadate in the presence of actin,
and the amount of [3H]ADP on M9bIQ4 co-sedimented with actin was determined. As shown in Fig. 9B, nearly 100% of actin-bound M9bIQ4 trapped [3H]ADP in the presence of Vi, whereas little [3H]ADP was trapped in the absence of Vi. These results suggest that M-ADP state of M9bIQ4 has high affinity for actin. The actin-bound M9bIQ4 was plotted against actin concentration to obtain $K_a$ of 1.0 μM (Fig. 9C).

**FIGURE 10. Model of processive movement of single-headed myosin IX.** A, steady-state distribution of the actomyosin IX intermediates. Simulations were performed using experimentally determined kinetic constants (Table 2) according to Scheme 1. B, proposed model for the movement of single-headed myosin IX (see “Discussion” for description).
DISCUSSION

Several critical features of the ATP hydrolysis mechanism of myosin IX, a single-headed processive myosin, were found. One is that the hydrolysis step is the rate-determining step for myosin IX ATPase reaction. All previously characterized myosin shows a rapid ATP hydrolysis step (16, 17, 20, 30, 31, 36), and this is the first report of myosin having the hydrolysis step as the rate-determining step of the ATPase cycle. In other word, myosin IXb spends the majority of its cycling time in a prehydrolysis state. It has been suggested that there is a catalytic water molecule adjacent to the Ser236 (residue number is based on the sequence of Dictostelium myosin II) at the Switch1 loop that attack the β-γ phosphoester bond to hydrolyze ATP (37). Therefore, it is likely that the position of the catalytic water molecule in the active site of myosin IX is a little distal from the β-γ phosphoester bond of ATP, thus causing slow ATP hydrolysis, which results in a long life of prehydrolysis state. The conserved amino acid residues in the three loops critical for constituting the active site of myosin (i.e. P-loop, Switch 1 loop, and Switch 2 loop) are well conserved in myosin IX, and it is likely that the slow hydrolysis is due to the difference in the overall conformation of the motor domain of myosin IX.

Another unique feature is the extremely slow ATP-induced dissociation rate of myosin IXb from actin. Because the ATP hydrolysis step is slow, the observed slow dissociation of myosin IXb from actin in the presence of ATP is due to the slow rate constant of $k_{d}$. The ATP-induced dissociation rate of myosin IX from actin ($k_{d}$) was 100-fold slower than other myosins (250–1500 s$^{-1}$), and consistently the affinity of myosin IX for actin in the presence of ATP is much higher than known myosin. Although the M·ATP state of myosin V shows high affinity to actin (4 μmol) (38), AM·ATP is not populated during the ATPase cycle for myosin V, because myosin V dissociates from actin quickly (>750 s$^{-1}$) upon ATP binding, and the hydrolysis ($k_{d} + k_{d}$) of myosin V is fast (750 s$^{-1}$) in the absence of actin. In contrast, slow hydrolysis of myosin IX in the absence of actin allows myosin IX to stably form the M9·ATP state. Furthermore, the slow dissociation of M·ATP from actin makes it possible to stably form AM·ATP at the saturated actin condition. Because of these features, we concluded that the major ATP hydrolysis pathway is the actin-attached pathway for myosin IX.

It has been thought that M·ATP is a weak actin binding state for all myosin so far reported and rapidly dissociates from actin. The present findings suggest that M·ATP state of myosin IX is not a conventional weak actin binding state. The affinity of M9·ATP for actin is much higher than other myosin so far reported. However, the affinity of M9·ATP to actin ($K_{0}$) is still weaker than those of M9 (K$K_{0}$) and M9·ADP (K$K_{0}$) states, and the dissociation rate of M·ATP of myosin IX from actin is faster than the conversion of M·ATP to M·ADP-P. Therefore, it is anticipated that M·ATP dissociates from actin for a several times before the formation of M·ADP-P, although the dissociated M·ATP quickly reassociates with actin at saturated actin condition. This suggests that the M·ATP state does not bind actin tightly enough to produce the movement, but its affinity is high enough to prevent myosin from diffusing away from actin.

Whereas the structural component responsible for this unique actin binding property of myosin IX is not known, it is plausible that the large insertion at the loop 2 region may play a role in the strong affinity of myosin IX to actin in the presence of ATP because the large insert of myosin IX at the site of loop 2 is rich in arginine and lysine residues. Mutational studies have revealed that the positive residues in loop 2 play a significant role in the affinity for actin in the presence of ATP (39–44). This unique large insertion of myosin IX may serve as an actin-anchor site to prevent myosin IX from free diffusion from actin filaments in the presence of ATP.

Unlike previously known myosin, myosin IX has a little (~1.3 times) activation of steady-state ATPase rate by actin (Fig. 2). This is not because the M9bIQ4 in the preparation is dead and we think that the presence of dead myosin is small, because 1) the $V_{max}$ values are consistent among the number of independent preparations, and 2) the M9bIQ4 preparation well supported in vitro actin gliding (5), and it is known that the presence of a relatively minor fraction of dead myosin in the preparation abolishes the in vitro actin gliding activity. The reason for the apparent low actin activation is that the rate-determining step of myosin IX is the ATP hydrolysis step, unlike other myosins in which the actin activation is due to the marked acceleration of the product release steps. The hydrolysis step could be rate-limiting for myosin II at non-physiological ionic conditions, and it was shown that actin did not activate the ATPase activity in such a condition (19).

Based upon the kinetic parameters determined in this study, we calculated the steady-state distribution of intermediates during ATPase cycle at the physiological nucleotide concentration (18) and the saturated actin concentration (Fig. 10A). The AM·ATP state is the predominant intermediate and populates 82% in this state. Myosin IX populates at the strongly binding AM·ADP state with 11% during the ATPase cycle. This indicates that myosin IX is a low duty ratio motor in which the population of the strong actin binding intermediate is less than 50%. It has been thought that the high duty ratio (>50%) is required for the two-headed processive motors (16, 17). Nevertheless, the recent studies have revealed that myosin IX moves processively on actin filaments (4, 5).

How does a single-headed myosin IX having low duty ratio move processively? It is shown by using an actin-gliding assay that one molecule of myosin IX is sufficient to translocate the actin filament for the entire length thus processive (5). Based upon the present findings, we propose the following model (Fig. 10B). Myosin IX in the presence of ATP forms the AM·ATP intermediate as a predominant intermediate. Upon the formation of this intermediate, the binding between the head of myosin IX and actin at the actin/myosin interface is weakened, resulting in partial dissociation from actin. However, the head is kept in close proximity to actin, presumably due to the presence of loop 2/large unique insertion of myosin IX (Fig. 10B, b). There is enough freedom for the myosin head to find the next location of the binding site along with the actin filament before complete dissociation from the filament (Fig. 10B, c). ATP hydrolysis occurs while myosin IX interacts with actin, but it is not tight enough to produce the reverse power stroke (Fig. 10B, d). After ATP hydrolysis, P$_{i}$ is quickly released to form a strong actin binding form, and a power stroke takes place (Fig. 10B, e). Some communication between the loop 2/insertion and the motor domain may be required that allows the motor domain to move along actin to the next binding site. Further studies are required to clarify the detailed molecular mechanism of the movement of myosin IX.

Addendum—After this manuscript was submitted, Nalavadi et al. (45) reported the actomyosin IXb ATPase activity using the full-length rat myosin IXb. The rate constants of several key steps of the ATP hydrolysis cycle are not reported in the paper by Nalavadi et al. (45), and therefore, we cannot precisely discuss the ATP hydrolysis mechanism with their results. For instance, the ADP off step from actomyosin IX, a critical step to estimate the duty ratio, was not reported. However, similarities and dissimilarities between the two papers can be addressed. The steady state activity of their construct is similar to the present study at similar temperature (20–25 °C), suggesting that the majority of the myosin preparations in both reports are active. On the other hand, the basal ATPase activity reported by Nalavadi et al. (45) was significantly lower than the value obtained in the present study. The difference could be due to the difference in the species or the presence of
the tail domain. As described in the Introduction, the tail domain may play an important role in determining the directionality, and further studies are required for clarifying the function of the tail domain of myosin IXb on its motor properties. They also concluded that the rate-limiting step of the ATP hydrolysis cycle is the ATP hydrolysis step, although the experiment was done only in the absence and not in the presence of actin. On the other hand, the rate of ATP binding determined by Nalavadi et al. (45) was much slower than that obtained in the present study and the rate was saturated at low ATP to yield the maximum ATP binding rate of 3 s\(^{-1}\). The reason for the difference is unclear, but it is possibly due to the presence of the tail domain or the difference in the species.

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