Mutations of myosin VIIA cause deafness in various species from human and mice to Zebrafish and Drosophila. We analyzed the kinetic mechanism of the ATPase cycle of Drosophila myosin VIIA using a single-headed construct with the entire neck domain. The steady-state ATPase activity (0.06 s\(^{-1}\)) was markedly activated by actin to yield \(V_{\text{max}}\) and \(K_{\text{ATPase}}\) of 1.72 s\(^{-1}\) and 3.2 \(\mu\)M, respectively. The most intriguing finding is that the ATP hydrolysis predominantly takes place in the actin-bound form (actin-attached hydrolysis) for the actomyosin VIIA ATPase reaction. The ATP hydrolysis rate was much faster for the actin-attached form than the dissociated form, in contrast to other myosins reported so far. Both the ATP hydrolysis step and the phosphate release step were significantly faster than the entire ATPase cycle rate, thus not rate-determining. The rate of ADP dissociation from actomyosin VIIA was 1.86 s\(^{-1}\), which was comparable with the overall ATPase cycle rate, thus assigned to be a rate-determining step. The results suggest that Drosophila myosin VIIA spends the majority of the ATPase cycle in an actomyosin:ADP form, a strong actin binding state. The duty ratio calculated from our kinetic model was \(\approx 0.9\). Therefore, myosin VIIA is classified to be a high duty ratio motor. The present results suggested that myosin VIIA can be a processive motor to serve cargo trafficking in cells once it forms a dimer structure.

Myosins are motor proteins that interact with actin filaments and convert the energy from ATP hydrolysis to produce a mechanical force. Myosins are classified based upon phylogenetic sequence comparisons of the motor domain and divided into at least 18 classes (1–3). Class VII myosins have been found in a variety of organisms from Dictostelium and Drosophila to mammals (4–7). There are two myosin VII genes identified in vertebrates and Drosophila, myosin VIIA and myosin VIB. Myosin VIIA has received a great deal of interest because it was identified as a responsible gene of human Usher syndrome type 1B (USH1B) (8), and two forms of nonsyndromic deafness, DFNB2 and DFNA11 (9–11). Quite recently, it was reported that myosin VIIA mutations lead to deafness in Drosophila (12). However, the reason why the lack of myosin VIIA causes these abnormalities is unknown, but obviously, this is due in part to the lack of knowledge regarding the myosin VIIA function at a molecular basis.

The N-terminal domain of myosin VIIA is a conserved motor domain, and there are no obvious large extensions or insertions in this domain. The neck domain contains five IQ motifs that are thought to be a light chain binding site. Because calmodulin is co-purified with myosin VIIA (13), it is thought that myosin VIIA has calmodulin as its light chain subunit. The tail domain consists of a proximal segment of coiled-coil domain followed by a globular domain. Because of the presence of the coiled-coil domain, it has been assumed that myosin VIIA exists as a dimer, i.e. two-headed structure. However, there is no hard evidence showing that myosin VIIA is a two-headed myosin. The tail of myosin VIIA contains two large repeats, each containing a MyTH4 domain and a talin homology domain (14). Although the function of MyTH4 domain is obscure, it has been proposed that this domain may function as a cargo-binding site. The talin-like domain is similar to domains in talin, ezrin, moesin, and other proteins. Because of the presence of these domains, myosin VIIA has been thought to be associated with membranes or vesicles.

Mammalian myosin VIIA is exclusively expressed along the stereocilia in the sensory hair cells (15, 16). It has been hypothesized that myosin VIIA may play a role in the assembly of the stereocilia into a bundled array, thus maintaining rigidity during the movement of the bundles. Myosin VIIA also serves as a transporter in the pericellular necklace of the hair cell (15) and photoreceptor cell (17). On the other hand, myosin VIIA plays an important role in phagocytosis in the photoreceptor cell (18) and in Dictyostelium (19). These cell biological studies have suggested the two potential functions, i.e. cargo transport and contraction.

One critical issue for the physiological relevance of each motor protein in diverse cellular motile systems is the processivity of each motor protein. It is known that conventional myosin is a typical nonprocessive motor in which the motor protein dissociates from the track (actin) during each cross-bridge cycle. On the other hand, a processive motor such as myosin Va, which can move on actin filaments for a long distance without dissociation (20–23), is suitable for cargo transport. It is unknown whether or not myosin VIIA is a processive motor.

The motor function of myosin VIIA at a molecular basis is largely unknown, and the aim of this study was to clarify the mechanoenzymatic characteristics of myosin VIIA by analyzing the kinetic mechanism of actomyosin VIIA ATPase. In particular, we attempted to determine the duty ratio that is closely related to the processivity of the motor proteins. Our results suggest that myosin VIIA is a high duty ratio motor.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes and modifying enzymes were purchased from New England Biolabs (Beverly, MA). Purine nucleoside phosphorylase, 7-methylguanosine, phosphoenolpyruvate, and pyruvate kinase were obtained from Sigma. Actin was prepared from rabbit skeletal muscle according to Spudich and Watt (24). Pyrene-actin was prepared as described (25). 7-Diethylamino-3-(((2maleimidyl)ethyl)amino) carbonyl)coumarin-labeled phosphate-binding protein (MDCC-PBP)\(^2\)

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\(^2\) The abbreviations used are: MDCC-PBP, 7-diethylamino-3-(((2maleimidyl)ethyl)amino) carbonyl)coumarin-labeled phosphate-binding protein; MOPS, 4-morpholinepropane-sulfonic acid; mant, N-methylanthraniloyl; A, actin; M, myosin; T, ATP; D, ADP.
was prepared as described (26, 27). Recombinant calmodulin was expressed in Escherichia coli and purified as described previously (28).

Cloning, Expression, and Purification of Drosophila Myosin VIIA Protein—Drosophila myosin VIIA cDNA was obtained from Drosophila total RNA by using reverse transcriptase-coupled PCR. The nucleotide sequence was determined by direct DNA sequencing to confirm the authenticity of the DNA sequence of the clone. The cDNA fragment encoding Met1–Glu926 was subcloned into modified pFastBacHT baculovirus transfer vector containing a FLAG tag sequence at the 5’-end of the polylinker region. This construct (DM7AIQ5) encodes the entire motor domain plus all five IQ motifs of myosin VIIA. To express the recombinant DM7AIQ5 protein, Sf9 cells (about 1 × 10^9 cells) were co-infected with two viruses expressing the DM7AIQ5 heavy chain and calmodulin. The infected cells were cultured for 3 days at 28 °C. Cells were harvested and washed with phosphate-buffered saline and 5 mM EGTA. Cells were then lysed with sonication in 20 ml of lysis buffer (30 mM MOPS-KOH (pH 7.5), 150 mM KCl, 2 mM MgCl_2, 1 mM MgATP, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml trypsin inhibitor). After centrifugation at 100,000 × g for 30 min, the supernatant was incubated with 300 μl of anti-FLAG M2 affinity resin in a 50-ml conical tube on a rotating wheel for 1 h at 4 °C. The resin suspension was then loaded on a column (1 × 10 cm) and washed with 30 ml of buffer A (20 mM MOPS-KOH (pH 7.5), 50 mM KCl, 2 mM MgCl_2, 1 mM EGTA, 5 μg/ml leupeptin, and 0.5 mM dithiothreitol). DM7AIQ5 was eluted with buffer B containing 0.1 mg/ml FLAG peptide. The DM7AIQ5 was dialyzed against buffer B (50 mM KCl, 20 mM MOPS-KOH (pH 7.5), 2 mM MgCl_2, 1 mM EGTA, and 1 mM dithiothreitol). The purified DM7AIQ5 was stored on ice and used within 2 days. 0.1–0.2 mg of the purified protein had an apparent molecular mass of 108 kDa and was co-purified with a entire neck domain (Met1–Glu926). We found that this construct (DM7AIQ5) is a single-headed structure based upon the molecular mass estimated by gel filtration analysis and electron microscopic observation (data not shown). DM7AIQ5 was used in this study to avoid the possible complexity because of the head-head interaction. DM7AIQ5 and calmodulin were co-expressed in Sf9 cells and purified with the anti-FLAG M2 affinity resin column. The isolated DM7AIQ5 had an apparent molecular mass of 108 kDa and was co-purified with a low molecular mass peptide (Fig. 1A). The mobility of the low molecular mass peptide on an SDS-PAGE increased with Ca^{2+}, suggesting that it is calmodulin (data not shown).

Steady-state ATPase Activity of DM7AIQ5—The steady-state ATPase activity of DM7AIQ5 was markedly activated by actin. Fig. 1B shows the ATPase activity of DM7AIQ5 as a function of actin concentration. The ATPase activities at various actin concentrations were fitted with a Michaelis-Menten equation, giving V_{max} and K_{ATPase} of 1.72 ± 0.12 s^{-1} and 3.2 ± 1.1 μM, respectively.

MgATP Binding to DM7AIQ5 and Acto-DM7AIQ5—Fig. 2 shows the mant-ATP concentration dependence of the rate of mant-ATP binding to DM7AIQ5. The time course of an increase in the fluorescence intensity was fitted with single exponential kinetics for both DM7AIQ5 and acto-DM7AIQ5 (Fig. 2, insets). The observed rate constants showed a linear dependence on the mant-ATP concentration. Based upon the tangent of the slope, a second order rate constant (k_{2}) of 3.4 ± 0.2 μM^{-1} s^{-1} for DM7AIQ5 was obtained. Most interestingly, the mant-ATP binding rate was markedly decreased by actin to yield a second order rate constant (k_{2}) of 0.47 ± 0.02 μM^{-1} s^{-1}. Because the y intercepts reflect the rate of the reverse reaction of the ATP binding step, k_{2} and k_{2} were estimated to be <0.3 and 0.58 ± 0.04 s^{-1}, respectively.

MgATP-induced Dissociation of Acto-DM7AIQ5—It is known that myosin quickly dissociates from actin upon the binding of ATP. The kinetics of ATP-induced dissociation of acto-DM7AIQ5 was monitored by measuring the ATP-induced change in the light-scattering intensity of acto-DM7AIQ5. The decrease in the light-scattering intensity because of the dissociation of DM7AIQ5 from actin followed single exponential kinetics (Fig. 3, inset). The obtained apparent rate constants showed a hyperbolic saturation curve against ATP concentration (Fig. 3). The initial tangent of the ATP dependence of the hyperbola repre-
FIGURE 2. Mant-ATP binding to DM7AIQ5 and acto-DM7AIQ5. The experiment was done in the buffer containing 50 mM KCl, 20 mM MOPS (pH 7.5), 2 mM MgCl₂, 1 mM EGTA, and 0.2 mg/ml calmodulin at 25 °C. A. In the absence of actin, 0.2 μM DM7AIQ5 was mixed with various concentrations of 2'-deoxy-mant-ATP. The second order rate constant for mant-ATP binding (k_{cat}k_{m}/K_{m}) was 3.4 ± 0.2 μM⁻¹s⁻¹. Inset shows a typical recording of the binding of mant-ATP (2 μM) to DM7AIQ5. The solid line is the best fit to single exponential kinetics with k_{obs} of 5.5 s⁻¹. B. 0.2 μM DM7AIQ5 in the presence of 0.2 μM actin was mixed with various concentrations of 2'-deoxy-mant-ATP. The second order rate constant (k_{cat}k_{m}/K_{m}) of 0.47 ± 0.02 μM⁻¹s⁻¹ and k_{obs} of 0.58 ± 0.04 s⁻¹ from the y intercept was obtained. Inset shows a typical recording of the binding of mant-ATP (2 μM) to acto-DM7AIQ5. The solid line is the best fit to single exponential kinetics with k_{obs} of 1.4 s⁻¹. The error bars represent the S.E. from three to six independent experiments.

FIGURE 3. ATP-induced dissociation of acto-DM7AIQ5. Change in the light-scattering intensity of acto-DM7AIQ5 was monitored after mixing acto-DM7AIQ5 (0.35 μM DM7AIQ5 + 0.35 μM actin) with various concentrations of MgATP. The second order rate constant for ATP binding can be estimated from the initial slope of the linear fit, and the obtained value (k_{cat}k_{m}/K_{m} = 0.35 ± 0.04 μM⁻¹s⁻¹) was consistent with the result obtained in Fig. 2. Inset shows time course of light-scattering change after mixing acto-DM7AIQ5 with 5 μM MgATP. The solid line is the best fit to single exponential kinetics with k_{obs} of 2.0 s⁻¹. The experimental conditions are as described in Fig. 2. The error bars represent the S.E. from three to six independent experiments.
Kinetic Mechanism of Drosophila Myosin VIIA

TABLE 1
Kinetic parameters of DM7AIQ5 ATPase cycle and comparison with myosin VIIB

| Steady state | Signal | Drosophila VIIA | Drosophila VIIB* | Mouse VIIB* |
|--------------|--------|-----------------|------------------|-------------|
| $v_0 (s^{-1})$ | 0.057 ± 0.041 | 0.02 | 0.03 | |
| $v_{max} (s^{-1})$ | 1.72 ± 0.12 | 8.4 | 1.17 | |
| $K_{ATP} (\mu M)$ | 3.2 ± 1.1 | 39 | 0.66 | |

ATP binding

| $K_i (\mu M)$ | Mant-ATP | Tryptophan | Light-scattering | Pyrene-actin | Mant-ATP | Mant-ATP |
|---------------|----------|------------|-----------------|-------------|----------|----------|
| $k_{i-2} (s^{-1})$ | 3.4 ± 0.2 | 2.6 ± 0.2 | 0.47 ± 0.02 | 0.35 ± 0.04 | 0.36 ± 0.05 | 0.35 ± 0.04 |
| $k_{i-2} (s^{-1})$ | 3.4 | 4.7 | 2.2, 0.46 | 1.1 | 1.3, 0.31 | 0.58 ± 0.04 |

ATP hydrolysis

| $k_{-3} + k_{-3} (s^{-1})$ | Tryptophan | Quenched-flow | Quenched-flow | Quenched-flow |
|--------------------------|------------|---------------|---------------|---------------|
| $k_{i-3}$ | 12.6 ± 0.9 | 13.2 ± 3.4 | 0.53 ± 0.05 | 0.53 ± 0.05 |
| $k_{i-3} + k_{-3} (s^{-1})$ | >50 | >50 | >50 | >50 |

Phosphate release

| $k_{-4} (s^{-1})$ | MDCC-PBP | Quenched-flow | $k_{-4} (s^{-1})$ | MDCC-PBP |
|-----------------|----------|---------------|-----------------|----------|
| $k_{-4} (s^{-1})$ | 0.067 ± 0.005 | 0.064 ± 0.025 | 0.17 ± 0.02 | 0.17 ± 0.02 |
| $k_{-4} (s^{-1})$ | 0.027 | 0.027 | 0.027 | 0.027 |
| $k_{-4} (s^{-1})$ | >40 | >40 | >40 | >40 |

ADP binding

| $k_{-5} (s^{-1})$ | Mant-ADP cold chase | Mant-ADP binding | Mant-ADP | Light-scattering | Mant-ADP cold chase | Mant-ADP binding | Mant-ADP |
|-----------------|-----------------|-----------------|----------|-----------------|-----------------|-----------------|----------|
| $k_{-5} (s^{-1})$ | 2.3 ± 0.1 | 1.8 ± 0.3 | 3.7 ± 0.1 | 0.62 ± 0.03 | 0.86 ± 0.03 | 2.1 ± 0.5 | 2.3 ± 0.5 |
| $k_{-5} (s^{-1})$ | 9 | 23 | 3 | 3.8 | 9.8 | 9.6 | 16 |
| $k_{-5} (s^{-1})$ | >10 | >10 | >10 | >10 | >10 | >10 | >10 |
| $k_{-5} (s^{-1})$ | 4.7 ± 0.2 | 4.7 ± 0.1 | 4.7 ± 0.2 | 4.7 ± 0.2 |
| $k_{-5} (s^{-1})$ | 2.7 | 2.7 | 2.7 | 2.7 |
| $k_{-5} (s^{-1})$ | 5.2 | 5.2 | 5.2 | 5.2 |

Actin binding

| $k_{-6} (s^{-1})$ | Pyrene-actin | Pyrene-actin | Pyrene-actin | Pyrene-actin |
|-----------------|-------------|-------------|-------------|-------------|
| $k_{-6} (s^{-1})$ | 0.0043 | 0.005 | 0.0046 | 0.0046 |
| $k_{-6} (s^{-1})$ | 1.2 | 0.042 | 0.035 | 0.0045 |
| $k_{-6} (s^{-1})$ | 0.054 | 0.043 | 0.04 | 0.043 |
| $k_{-6} (s^{-1})$ | 0.026 | 0.026 | 0.026 | 0.026 |

* Data are from Ref. 41.
* Data are from Ref. 42.
* Pyrene-actin.

presents a second order rate constant of ATP binding to acto-DM7AIQ5 ($K_iK_{-i}$. The value (0.35 ± 0.04 μM$^{-1}$ s$^{-1}$) was consistent with that obtained by measuring directly using mant-ATP (Fig. 2). The results suggest that the mant-moietiy does not significantly influence the ATP binding rate to DM7AIQ5. The observed rate constants were saturated at >200 s$^{-1}$, which was more than 100-fold larger than the $V_{max}$ value of the steady-state ATPase activity of DM7AIQ5. Pyrene-labeled actin was also employed to monitor the ATP-induced population of the weakly bound acto-DM7AIQ5 state. The fluorescence intensity of pyrene-actin increased upon the addition of ATP to pyrene-acto-DM7AIQ5. From the time course of the observed fluorescence transition, the apparent rate constants were determined at various ATP concentrations. The results showed a hyperbolic dependence against ATP concentration, and the ATP dependence was almost identical to that obtained from the change in light scattering (Table 1). These results suggest that the ATP binding step and the following isomerization step ($k_{i-3}$) are not rate-limiting for acto-DM7AIQ5 ATPase cycle, and the dissociation of acto-DM7AIQ5 ($k_{-3}$) pathway is a rapid process.

Enhancement of Intrinsic Tryptophan Fluorescence Intensity—

Drosophila myosin VIIA contains a conserved tryptophan residue (Trp$^{378}$) located at the rigid relay loop, which is correlated to Trp$^{310}$ of skeletal muscle myosin II, a residue predominantly responsible for the ATP-induced conformational change of the myosin motor domain. The time course of an increase of the intrinsic tryptophan fluorescence intensity upon the addition of ATP followed single exponential kinetics (Fig. 4, inset). The ATP dependence of the apparent rate constants showed a hyperbolic relationship to yield a maximum rate constant of 12.6 ± 0.9 s$^{-1}$ (Fig. 4). The ATP binding rate constant estimated from the initial slope of the hyperbola ($K_iK_{-i}$) was 2.6 ± 0.2 μM$^{-1}$ s$^{-1}$. This value was in good agreement with the value obtained using mant-ATP (Fig. 2). We assumed that the observed maximal rate constant of 12.6 s$^{-1}$ corresponds to the rate of the ATP hydrolysis step ($k_{i-3} + k_{-3}$), because this value is much less than the ATP binding rate at a high ATP concentration.

Quenched-flow Measurements of ATP Hydrolysis by Acto-
DM7AIQ5—Fig. 5A shows the time course of the Pi burst of DM7AIQ5 ATPase reaction measured by a quenched-flow experiment. Single turnover experiments were carried out in which the active site concentration exceeded the substrate concentration. Because all given ATP is bound to the ATP-binding site of DM7AIQ5, the equilibrium of the ATP hydrolysis step can easily be determined by the fraction of the fast Pi burst phase. The time course of the Pi burst followed double exponential kinetics. The fast phase corresponds to the initial rapid ATP hydrolysis in which ATP binding is rate-limiting. The slow phase (0.64 ± 0.025 s$^{-1}$) represents the apparent Pi release rate ($k_{-4 obs}$). From the fractional amplitudes of the fast and slow phase, the Pi burst size was estimated to be 0.34 ± 0.03, and the equilibrium constant of ATP hydrolysis ($K_i$) was calculated to be 0.53 ± 0.05. The result indicates that the equilibrium of the ATP hydrolysis step is significantly
FIGURE 4. ATP-induced intrinsic tryptophan fluorescence change of DM7AIQ5. 0.2 mM DM7AIQ5 was mixed with various concentrations of MgATP, and the tryptophan fluorescence change was monitored. The observed rates ($k_{obs}$) were saturated at 12.6 ± 0.9 s$^{-1}$ ($k_{+3} + k_{-3}$). The second order rate constant for ATP binding can be estimated from the initial slope of the linear fit, and the obtained value ($k_{+3} = 2.6 ± 0.2$ μM$^{-1}$ s$^{-1}$) was consistent with that obtained in Fig. 2. Inset shows a typical recording of intrinsic tryptophan fluorescence change after mixing DM7AIQ5 with 2 μM MgATP. The solid line is the best fit to single exponential kinetics with $k_{obs}$ of 4.8 s$^{-1}$. The experimental conditions are as described in Fig. 2. The error bars represent the standard error from three to six independent experiments.

FIGURE 5. Kinetics of ATP hydrolysis step of DM7AIQ5. A, single turnover experiment. 0.4 μM DM7AIQ5 was mixed with 0.2 μM $[^{32}$P]ATP, and the fraction of hydrolyzed ATP was plotted against time. The time course was fitted to double exponential kinetics. The rate constant of the burst phase ($k_{obs}$ = 15.5 s$^{-1}$) with a fractional amplitude (−$A_{0obs}$/(−$A_{0obs}$ + $A_{obs}$)) of 0.31 was obtained. The slow phase of the reaction had $k_{obs}$ of 0.06 s$^{-1}$. B, multiple turnover experiment. $P_i$ burst was measured after mixing 0.8 μM DM7AIQ5 with 100 μM $[^{32}$P]ATP in the presence (closed circle) or absence (open circle) of 10 μM actin. The apparent rate constants of ATP hydrolysis in the presence and absence of actin were 44.6 and 12.0 s$^{-1}$, respectively. The estimated $P_i$ burst sizes were 0.92 and 0.40 in the presence and absence of actin, respectively. The experimental conditions are as described in Fig. 2. The error bars represent the S.E. from three independent experiments.

shifted to the pre-hydrolyzed form (MT). The rate of the ATP hydrolysis was also measured at a higher ATP concentration where multiple turnover of ATP hydrolysis takes place (Fig. 5B). The initial $P_i$ burst rate was 13.2 ± 3.4 s$^{-1}$ in the presence of 100 μM ATP. Because the ATP binding rate at this ATP concentration does not limit the rate of ATP hydrolysis, the obtained rate constant represents the rate of ATP hydrolysis ($k_{+3} + k_{-3}$). The observed value is consistent with the value obtained by the measurement of intrinsic tryptophan fluorescence change. It should be noted, however, that the rate constant of the ATP hydrolysis step is still significantly larger than the steady-state ATPase cycle rate, thus not limiting the overall ATPase rate.

We also measured the $P_i$ burst rate in the presence of actin (at 10 μM that is three times of the $K_{ATPase}$ value). Most interestingly, the $P_i$ burst rate was significantly larger than that in the absence of actin, and the $P_i$ burst rate of 43.6 ± 6.5 s$^{-1}$ was obtained at 100 μM ATP. Furthermore, the $P_i$ burst size in the presence of actin was nearly 1 and was much larger than that in the absence of actin. The results suggest that the ATP hydrolysis rate of the actin-bound form of DM7AIQ5 is much faster than that of the actin-dissociated form. This is quite different from the other myosins reported so far. It has been thought that the ATP hydrolysis rate is significantly decreased with bound actin (26), although the overall ATPase cycle rate is largely accelerated because of the enhancement of the rate-limiting product release steps. These results suggest that the actin-attached ATP hydrolysis is the major ATP hydrolysis pathway of actomyosin VIIA ATPase reaction.

Phosphate Release Rate—The rate of the phosphate release step was determined by measuring the fluorescence increase of the fluorescently labeled phosphate-binding protein (MDCC-PBP). Double-mixing single turnover experiments were carried out in which DM7AIQ5 was mixed with substoichiometric amounts of ATP, aged for 10 s to allow ATP binding and hydrolysis, and then mixed with various concentrations of actin. Upon the release of phosphate, MDCC-PBP rapidly binds to the phosphate, and the observed fluorescence increase followed single exponential kinetics in the absence of actin (Fig. 6A, inset). The observed rate ($k_{+4obs}$) of 0.067 ± 0.005 s$^{-1}$ was consistent with the rate of the slow phase obtained from the quenched-flow experiment (Fig. 5A). In the presence of actin, the time course of the fluorescence increase followed double exponential kinetics. At 50 μM actin, the fast and slow apparent rate constants were 24.5 ± 1.1 and 0.91 ± 0.31 s$^{-1}$, respectively (Fig. 6A).

The fast phase was actin concentration-dependent, which reflects the actin re-binding step as shown in the kinetics scheme (Scheme 1). The observed rate constant increased with actin concentration, and a second order rate constant was determined to be 0.49 ± 0.02 μM$^{-1}$ s$^{-1}$. The maximum $P_i$ release rate from acto-DM7AIQ5-ADP-Pi was estimated to be >50 s$^{-1}$ based upon the lack of curvature in Fig. 6B. The fractional amplitude of the slow phase was ~15% of the total amplitude, and the rate was actin concentration-independent in contrast to the fast phase...
A similar slow phase was previously observed for myosin X (31, 32). Kovacs et al. (31) proposed that the slow phase is derived from an “actin-attached hydrolysis” (AMT-AMDP transition; where A is actin; M is myosin; T is ATP; and D is ADP). On the other hand, Homma and Ikebe (32) found that the slow phase is because of ATP re-binding to myosin X, because the rate of the slow phase was linearly increased with myosin concentration.

At the ATP concentration in this experiment (0.2 μM), the rate constant of the forward reaction is \( \sim 0.7 \text{ s}^{-1} \). Furthermore, from the rate constant of \( \leq 0.3 \text{ s}^{-1} \) for the reverse reaction \( (k_{-3}) \) and the equilibrium constant of the ATP hydrolysis step \( (K_a = 0.53) \), the observed contribution of the slow phase to the total phase can be estimated to be \( \sim 20\% \). Therefore, we conclude that the actin-independent slow phase observed in Fig. 6A is because of the ATP re-binding step of DM7AIQ5. The obtained results indicate that the phosphate release step \( (>50 \text{ s}^{-1}) \) is not the rate-limiting step of acto-DM7AIQ5 ATPase cycle.

Kinetics of ADP Binding to and Dissociation from Acto-DM7AIQ5—We employed mant-ADP as a probe to determine the rate of ADP binding to acto-DM7AIQ5. Both in the presence and absence of actin, the fluorescence intensity of mant-ADP increased after mixing with DM7AIQ5 because of the binding of mant-ADP to the active site of DM7AIQ5. The time course of an increase in fluorescence intensity followed single exponential kinetics (Fig. 7, A and B, insets), and the observed apparent rate constants were linearly increased with mant-ADP concentration in both the absence and presence of actin (Fig. 7). In the absence of actin, a second order rate constant \( (k_{-5}) \) was \( 3.7 \pm 0.1 \mu\text{M}^{-1} \text{s}^{-1} \) (Fig. 7A). The y intercept of the mant-ADP dependence gave the rate constant of the mant-ADP release, which was determined to be \( 2.3 \pm 0.5 \text{ s}^{-1} \) was obtained from the y intercept (Fig. 7B). The rate of ADP dissociation from acto-DM7AIQ5 was also determined by measuring acto-DM7AIQ5 dissociation after the addition of ATP. In the presence of ADP, the rate of ATP-induced dissociation of acto-DM7AIQ5 is limited by ADP release. DM7AIQ5 and actin were mixed in the presence of excess ADP, and then the ATP-induced change in the light-scattering intensity was monitored. Fig. 8 shows the rate of ATP-induced dissociation of the acto-DM7AIQ5-ADP complex as a
The rate of actin binding to DM7AIQ5 was also measured in the presence of ADP. The time course of the change in pyrene fluorescence intensity followed single exponential kinetics. The observed rate constants showed linear actin concentration dependence to yield a second order rate constant ($k_{32}$) of 0.32 ± 0.02 $\mu M^{-1}s^{-1}$ (Fig. 9). From the $y$ intercept, the rate constant for actin dissociation ($k_{10}$) was estimated to be 0.030 ± 0.005 s$^{-1}$. The affinity of actin to DM7AIQ5·ADP ($K_{10}$) was calculated to be ~0.1 $\mu M$.

**DISCUSSION**

Overview—In the present study, we analyzed the enzyme kinetic mechanism of the actomyosin VIIA ATPase cycle. The most intriguing finding is that the ATP hydrolysis predominantly takes place in the actin-bound form (actin-attached hydrolysis). The ATP hydrolysis step and the Pi release step are significantly faster than the entire ATPase cycle rate, thus not rate-determining. On the other hand, the rate of ADP release step is slow and comparable with the entire ATPase cycle.
rate. The results suggest that Drosophila myosin VIIA spends the majority of the ATPase cycle in AMT form, a strong actin binding state.

**Actin-attached Hydrolysis Pathway (AMT-AMDP)**—The most unique feature of the ATPase mechanism of Drosophila myosin VIIA is that the actin-attached hydrolysis is a major pathway for the ATPase cycle. This is based upon the following findings. First, the ATP hydrolysis rate (k_{3+} + k_{-3}) of 13 s\(^{-1}\) was slow in the actin-dissociated form of Drosophila myosin VIIA. This value was consistent between the two different measurements, i.e. P\(_i\) burst measurement and the change in intrinsic tryptophan fluorescence enhancement of Drosophila myosin VIIA. Quite interestingly, however, the P\(_i\) burst rate was much higher in the presence of actin. This is unusual for myosin. Because most myosins so far reported show fast actomyosin dissociation and slow actin-attached ATP hydrolysis rate, nearly all myosin goes through the actin-dissociated pathway; therefore, the observed P\(_i\) burst rate in the presence of actin is apparently the same as that in the absence of actin (34). The above finding for Drosophila myosin VIIA can be explained as follows. The actin-attached ATP hydrolysis rate is much faster than that of the actin-dissociated form. It is anticipated that AMT-MT is in fast equilibrium. Therefore, even if myosin VIIA is dissociated from actin upon ATP binding, the dissociated myosin VIIA quickly reassociates with actin (AMT), and the bound ATP is hydrolyzed. In other words, ATP hydrolysis hardly takes place in the actin-dissociated form of myosin VIIA. This view was confirmed by computer simulation of the overall ATP hydrolysis cycle pathway (see below).

Second, if the actin-dissociated hydrolysis is a major pathway, a slow phase of ~13 s\(^{-1}\) because of the ATP hydrolysis step (k_{3+} + k_{-3}) should be observed at high actin concentrations in the measurement of phosphate release because the equilibrium of the actin-dissociated ATP hydrolysis step (K\(_d\)) is significantly shifted to the pre-hydrolyzed form. However, the slow phase was not observed at high (>40 μM) actin concentrations (Fig. 6), suggesting that the pre-hydrolyzed form goes through other faster pathways (actin-attached hydrolysis) upon the addition of actin.

Third, if the ATPase cycle predominantly flows through actin-dissociated hydrolysis, V\(_{max}\) of the steady-state actin-activated ATPase activity calculated by computer simulation is ~1.1 s\(^{-1}\), where the overall ATPase cycle can be explained by the ADP off-rate (k_{-3}) and the equilibrium constant of the actin-dissociated ATP hydrolysis step (K\(_d\)). However, V\(_{max}\) measured experimentally (1.7 s\(^{-1}\)) was significantly faster than the calculated one, supporting the existence of faster pathways of the actin-attached ATP hydrolysis.

**The Kinetic Mechanism of Drosophila Myosin VIIA ATPase Cycle**—All rate constants and equilibrium constants obtained in the present study are summarized in Table 1. The overall steady-state ATPase rate of Drosophila myosin VIIA was markedly activated by actin. Although the ATP hydrolysis rate is slow without actin and significantly enhanced by actin as described above, the ATP hydrolysis rate (k_{3+} + k_{-3}) is still much faster than the overall basal ATPase cycle rate of Drosophila myosin VIIA. The basal steady-state ATPase rate is explained by the slow phosphate release rate (k_{-3}) and the unfavorable equilibrium of the ATP hydrolysis step (K\(_d\)). The basal steady-state ATPase activity (0.057 s\(^{-1}\)) agrees with the observed phosphate release rate of 0.067 s\(^{-1}\).

On the other hand, in the presence of actin, the phosphate release step is largely increased to be >50 s\(^{-1}\) and is not the rate-limiting step. As described above, ATP hydrolysis predominantly takes place through the actin-attached pathway. P\(_i\) burst rate in the presence of actin (k_{3+} + k_{-3}) at 100 μM ATP, where the rate of ATP binding step is ~47 s\(^{-1}\), can be estimated to be >50 s\(^{-1}\). Because P\(_i\) burst size in the presence of actin was >0.9, k_{-3} predominantly explains the P\(_i\) burst rate constant. All rate constants up to the formation of myosin VIIA-ADP are much higher than the entire ATPase cycle rate. On the other hand, the rate constant of the ADP release step (k_{-3}) was 1.87 s\(^{-1}\), which is comparable with V\(_{max}\) of the steady-state ATPase activity. Therefore, we concluded that the rate-determining step of the actomyosin VIIA ATPase reaction is the ADP release step.

Based upon the obtained rate constants and equilibrium constants of each elementary kinetic step of the actomyosin VIIA ATPase cycle, we calculated the steady-state ATPase activity under saturating ATP concentrations with the ATP-regeneration system as a function of actin concentration, and the result is shown by the broken line in Fig. 1B. The major kinetic pathway is shown in boldface in Scheme 1. The contribution of k_{-3}, [M], and [MD] to the overall ATPase rate was ignored for the simplicity of the simulation. The initial values, rates, and equilibrium constants employed in the simulation were as follows (where A is actin; M is myosin; T is ATP; D is ADP; and P is phosphate): [AM]\(_0\) = 20 nm; [AMT]\(_0\) = [M]T\(_0\) = [MDP]\(_0\) = [AMD]\(_0\) = [AMD]\(_0\) = 0 nm; K\(_d\)\(_T\) = 940 s\(^{-1}\); K\(_d\)\(_P\) = 50 μM (rapid equilibrium); k_{3+} = 4.5 s\(^{-1}\); k_{-3} = 8.5 s\(^{-1}\) (K\(_d\) = 0.53); k_{3+} = 400 s\(^{-1}\); K\(_d\) = 50 μM (rapid equilibrium); and K\(_d\)\(_P\) = 50 s\(^{-1}\), k_{3+} = 1.87 s\(^{-1}\). The V\(_{max}\) and K\(_{ATP}\) values calculated by this simulation were 1.77 s\(^{-1}\) and 2.1 μM, respectively. These values were in good agreement with the experimentally obtained values.
Duty Ratio and Processivity of Myosin VIIA—One of the most important issues to evaluate the physiological role of the motor proteins is the processivity, because it is thought that the processive motors are suitable for the cargo transporter in cells, while the nonprocessive myosin molecules can simultaneously interact with a single actin filament to produce a large force. It has been suggested that the processive movement of the two-headed myosin requires a duty ratio of greater than 0.5. The duty ratio of Drosophila myosin VIIA was calculated based upon the kinetic constants obtained in the present study to be -0.9. This value is higher than the duty ratio of a well known processive myosin Va, 0.7 (34), and comparable with that of myosin VI, ~0.9 (35).

The results suggest that myosin VIIA can be a processive myosin. A key question is whether or not myosin VIIA is a two-headed myosin. Although it has been shown that a single-headed construct of high duty ratio myosin Va can produce successive multiple steps, suggesting its continuous movement on actin (36), it is thought that the two-headed structure is critical for physiological long distance processive movement in cells. According to the coiled-coil prediction, it was thought that myosin VIIA has a short coiled-coil domain right next to the neck domain containing the IQ motifs (14). However, the length of the putative coiled-coil region is quite short, and it has been questioned whether this myosin forms a stable two-headed structure. Quite recently, Knight et al. (37) reported that the N-terminal side of the “coiled-coil” domain of myosin X forms a stable single α-helix and does not dimerize to form a coiled-coil structure. By sequence comparison of the coiled-coil domain of myosin X and myosin VIIA, it was suggested that the coiled-coil domain of myosin VIIA does not form a coiled-coil structure (37). Consistent with this notion, we found that the DM7AIQ5 construct having the coiled-coil region does not form a dimer structure according to electron microscopic observations (data not shown). Therefore, it is unlikely that the coiled-coil domain itself is sufficient to stabilize a dimer structure of myosin VIIA. Myosin VI has been reported as a high duty ratio motor, which has the rate-limiting ADP release (35). It is shown that myosin VI moves processively on actin filaments when it forms a dimer (38, 39). However, it becomes non-processive when it does not form a dimer (40). It is likely that the processivity of myosin VIIA depends upon its ability to form a dimer structure. Whether native myosin VIIA forms dimer or not is an important question, and further studies are required to answer this question.

Differences between Myosin VIIA and VIIIB—While this manuscript was in preparation, the kinetic mechanism of Drosophila myosin VIIB was reported independently from two groups (41, 42). There are several differences in the kinetic constants between myosin VIIA and VIIIB, which are summarized in Table 1. A major difference is that the ATP hydrolysis takes place in the actin-attached form for myosin VIIA, although myosin VIIIB hydrolyzes ATP in the actin-dissociated form. This is because of the marked difference in the rate of the ATP hydrolysis step (k_n + k_-) between the two types of myosin VII. At present, it is difficult to assign the residues responsible for this difference because a large number of residues are not conserved between myosin VIIA and VIIIB with 53–60% identity of the residues in the motor domain.

Implications for the Cellular Function—As described under the Introduction, the cell biological studies have suggested that myosin VIIA may serve as a force producer as well as a cargo transporter. It has been suggested that myosin VIIA may play a role in phagocytosis of outer segments of the photoreceptor cells by the pigmented epithelium that is thought to be an actin-based process (18). A role of myosin VII in phagocytosis was shown in Dictyostelium (19), and it was suggested that myosin VII is involved in the particle engulfment process requiring force generation. This view was supported by a recent study that shows abnormal phagocytosis of the photoreceptor outer segment disks membranes by retinal pigment epithelium lacking myosin VIIA (43). On the other hand, it was shown that myosin VIIA associates with melanosome via Slac-c/MyRIP and suggested that it transports melanosomes (44, 45). The association of myosin VIIA with lysosomes was also reported (46). The studies described above have suggested the two potential functions of myosin VIIA, i.e. cargo transport and contraction. For cargo movement, a processive motor is more suitable. On the other hand, production of force requires many motor molecules to interact on an actin filament simultaneously, and therefore a nonprocessive motor is rather suitable for this task.

The present studies reveal that the duty ratio of myosin VIIA is higher than 0.5, suggesting that myosin VIIA can be a processive motor, although a motor protein having a high duty ratio does not guarantee a processive movement (47, 48). This is closely related to the question whether myosin VIIA forms a two-headed structure. Although its short coiled-coil domain is less likely to be sufficient to stabilize the dimer formation, it is plausible that the large tail domain contributes to the dimer formation of myosin VIIA. Another possibility is that the myosin VIIA-binding proteins are involved in the monomer-dimer transition of myosin VIIA. In such a case, myosin VIIA forms a dimer when it binds to the particular target molecules such as the linker molecules to the vesicles at the tail domain of myosin VIIA. Further studies are required to clarify these questions.

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