Human Myosin III Is a Motor Having an Extremely High Affinity for Actin*

Received for publication, April 20, 2006, and in revised form, September 11, 2006. Published, JBC Papers in Press, October 1, 2006, DOI 10.1074/jbc.M603823200

Taketoshi Kambara, Shigeru Komaba, and Mitsuo Ikebe

From the Department of Physiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655

Myosin IIIA is expressed in photoreceptor cells and thought to play a critical role in phototransduction processes, yet its function on a molecular basis is largely unknown. Here we clarified the kinetic mechanism of the ATPase cycle of human myosin IIIA. The steady-state ATPase activity was markedly activated ~10-fold with very low actin concentration. The rate of ADP off from actomyosin IIIA was 10 times greater than the overall cycling rate, thus not a rate-determining step. The rate constant of the ATP hydrolysis step of the actin-dissociated form was very slow, but the rate was markedly accelerated by actin binding. The dissociation constant of the ATP-bound form of myosin IIIA from actin is submicromolar, which agrees well with the low $K_{act}$. These results indicate that ATP hydrolysis predominantly takes place in the actin-bound form for actomyosin IIIA ATPase reaction. The obtained $K_{act}$ was much lower than the previously reported one, and we found that the autophosphorylation of myosin IIIA dramatically increased the $K_{act}^*$ whereas the $V_{max}$ was unchanged. Our kinetic model indicates that both the actin-attached hydrolysis and the P$_i$ release steps determine the overall cycle rate of the dephosphorylated form. Although the stable steady-state intermediates of actomyosin IIIA ATPase reaction are not typical strong actin-binding intermediates, the affinity of the stable intermediates for actin is much higher than conventional weak actin binding forms. The present results suggest that myosin IIIA can spend a majority of its ATP hydrolysis cycling time on actin.

Class III myosin was originally found in Drosophila photoreceptor cells (1), and a majority of the cell biological work related to class III myosin has been done with the Drosophila system. Class III myosin was subsequently identified from humans (2, 3), striped bass (4), and Limulus (5). In vertebrate, two isoforms of class III myosin, myosin IIIA and myosin IIIIB, have been cloned (2, 3). Among them, most studies have been done with myosin IIIA. Both isoforms are highly expressed in the retina. Myosin IIIA is also expressed in inner hair cells, and it is responsible for progressive nonsyndromic hearing loss in humans (6). Immunohistochemical studies revealed that myosin IIIA is concentrated in the distal ends of rod and cone ellipsoid and colocalizes with the plus-distal ends of inner segment actin filament bundles, where actin forms the microvilli-like calycal processes (4). Interestingly, the transfection of green fluorescent protein-myosin IIIA into HeLa cells revealed that myosin IIIA localizes at the tip of filopodia (7), suggesting that myosin IIIA accumulates at the plus end of actin bundles. The major cytoskeletal structure of filopodia is the actin bundles, and the plus ends of the actin filaments are localized at the tip; therefore, the localization of myosin IIIA at the tip of filopodia suggests that this myosin traveled on actin filaments and accumulated at the end of the actin track. This is consistent with our result that myosin IIIA is a plus end-directed motor (8).

The physiological function of myosin III is still unknown, but there are several possibilities for the function of myosin III. Myosin III may play an important role in maintaining the structural integrity of microvillar apparatus of photoreceptor cells, since retinal degeneration occurs in ninaC mutant flies (9–11). On the other hand, recent studies have suggested that myosin III functions as a cargo carrier. It has been known that the two important signaling proteins show light-dependent translocation in photoreceptor cells of both vertebrate and invertebrate. One is G-protein transducin in vertebrate and $G_q$ in invertebrate. G-proteins mediate phototransduction signaling. Recent studies have revealed that transducin and $G_q$ translocate between the signaling compartments, and this plays a role in an adaptation mechanism (12–14). The other is visual arrestin that is responsible for termination of the photoresponse. Visual arrestin translocates in the direction opposite to the G-protein translocation (15–17). Although the mechanism of the translocation of these molecules is unknown, myosin III has been thought to be a candidate of the transporter of this process.

Like most other myosins, myosin III heavy chain consists of the motor domain, the neck domain, and the C-terminal tail domain. The most intriguing feature of myosin III is that it contains a protein kinase homology domain at the N-terminal side of the myosin homology domain. The protein kinase activity of this domain has been biochemically identified in our laboratory for ninaC (18) and human myosin IIIA (8). Although it had been questioned whether myosin III has motor activity, the motor function of human myosin IIIA was identified previously (8). Following the motor domain, there is a neck domain containing various numbers of IQ motifs that serve as a light chain/CaM$^2$ binding site.

---

* This work was supported by National Institutes of Health Grants AR048898, AR04856, and DC006103. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Dept. of Physiology, University of Massachusetts Medical School, 55 Lake Ave. N., Worcester, MA 01655. Tel.: 508-856-1954; Fax: 508-856-4600; E-mail: mitsuo.ikebe@umassmed.edu.

2 The abbreviations used are: CaM, calmodulin; mantATP, methylanthraniloyl-ATP; mantADP, methylanthraniloyl-ADP; HuM3MD, human myosin III motor domain; M3KD, the kinase domain of human myosin III; GST, glutathione S-transferase.
It has been shown that myosin IIIA interacts with CaM (4, 8). There is no coiled-coil region in the tail region, suggesting that myosin III is a single-headed structure.

Lin-Jones et al. (19) found that the tail domain of myosin IIIA is required for the localization at the rod calycal processes. Consistently, the tail domain is required for the localization of myosin IIIA to the distal filopodial tips. Furthermore, the tail domain of myosin IIIA co-localized with actin structures, suggesting that the tail domain may interact with actin (7). Supporting this notion, the short tail peptide conjugated to GST co-sedimented with F-actin in vitro. This raises a hypothesis that the actin binding site at the tail serves as a tethering site and plays a critical role in preventing myosin IIIA from dissociating from the actin track during the movement, thus supporting processive movement as is shown for the processive movement of kinesin (21).

However, the motor function of myosin III at a molecular basis is largely unknown. In the present study, we expressed the motor domain of human myosin IIIA using the baculovirus expression system and clarified the mechanoenzymatic characteristics of myosin IIIA by analyzing the actomyosin IIIA ATPase reaction. The results suggest that myosin IIIA is not a conventional high duty ratio motor, but it spends a majority of its ATP hydrolysis cycle time on actin.

**EXPERIMENTAL PROCEDURES**

**Reagents and Proteins**—Rabbit skeletal muscle actin was purified according to Spudich and Watt (22), and actin filaments were stabilized by phalloidin. Pyrene-labeled actin was purified according to Spudich and Watt (22), and actin filaments were inserted at the C terminus. The HuM3MD construct containing only the motor domain of human myosin IIIA (Fig. 1A). The HuM3MD bound with actin (7). Supporting this notion, the short tail peptide conjugated to GST co-sedimented with F-actin in vitro. This raises a hypothesis that the actin binding site at the tail serves as a tethering site and plays a critical role in preventing myosin IIIA from dissociating from the actin track during the movement, thus supporting processive movement as is shown for the processive movement of kinesin (21).

**Preparation of Recombinant Myosin III**—To create myosin III motor domain (HuM3MD), a fragment of human myosin IIIA cDNA encoding Met311–Lys1058 was subcloned into pFastBac1 vector (Invitrogen). A Myc tag and an octahistidine tag were inserted at the C terminus and subcloned into pFastBac1 vector. M3KD was purified by the same procedure with HuM3MD described above. The protein was snap-frozen in liquid nitrogen and stored at −80 °C.

**Actin Binding Assay**—Various concentrations of actin (0.3–1.5 μM) was mixed with 0.5 μM HuM3MD in buffer A containing 1 mM ATP, 20 units/ml pyruvate kinase, and 2 mM phosphoenolpyruvate and then incubated for 5 min at room temperature. The samples were centrifuged with 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, and then the band intensities were quantified using the ImageJ software to determine the percentage of HuM3MD bound to pelleted actin. The fraction of HuM3MD bound with actin (F) was plotted as a function of total actin concentration ([A]₀). The dissociation constant, Kₐ₀, was determined by fitting with Equation 1.
where \([M]_o\) represents total HuM3MD concentration, and \([AM] = [M]_o F\).

**Kinetic Experiments**—All transient kinetic experiments were done in buffer A at 25 °C using a Kin-Tek SF-2004 stopped flow apparatus with 150-watt mercury-xenon arc lamp. Fluorescence changes of mantATP and mantADP were measured by fluorescence energy transfer by exciting at 280 nm, and the emission was detected at 400 nm with a long pass filter (Oriel). Pyrene-actin was exited at 360 nm, and the emission was detected at 400 nm with a long pass filter. For 90° light scattering, the excitation beam was passed through a 400-nm interference filter.

Quenched flow measurements were performed with Kin-Tek RQF-3 apparatus (KinTek Corp.). Samples of HuM3MD or acto-HuM3MD were mixed with an equal volume of \([^{32}P]ATP\) in buffer A. After aging in the delay line, reactions were stopped by mixing it with a solution containing 0.3 n perchloric acid, and the liberated \(^{32}\)P was measured.

All of the transients shown are the average of 3–6 independent mixings. Single exponential data were fit to the equation \(y = c + a_1 (exp^{-k_{obs1} t}) + a_2 (exp^{-k_{obs2} t})\), where \(c\) is constant and \(a_1\) and \(a_2\) are the amplitude coefficients of reactions with rate constant \(k_{obs1}\) and \(k_{obs2}\), respectively. Kinetic modeling and simulation based upon Scheme 1 were performed using STELLA software version 8.1.1 (Isee Systems).

**RESULTS**

**Expression and Purification of Myosin III Construct**—We expressed and purified HuM3MD construct (Fig. 1) that contains only the motor domain. It has been shown that the kinase domain of myosin III autophosphorylates the C-terminal region of the motor domain (8). Thus, we could avoid the potential complexity arising from phosphorylation due to the presence of the N-terminal kinase domain by using the HuM3MD construct without the N-terminal kinase domain. We found that the affinity of CaM light chain for human myosin III heavy chain is relatively low, and the isolated myosin III construct having IQ motifs does not contain saturated amounts of CaM (data not shown). Therefore, we used the HuM3MD construct having the entire motor domain but no IQ domain to avoid complexity arising from the heterogeneity of the molecules in terms of bound CaM. It should be noted that HuM3IQ2, having two IQ motifs in addition to HuM3MD, showed similar \(K_{act}\) and \(V_{max}\) to those of HuM3MD in the presence of exogenous calmodulin (not shown). Our approach in this study allowed us to examine the basal kinetic mechanism of human myosin III ATPase cycle. The isolated HuM3MD had an apparent molecular mass of 90.6 kDa, and no small molecular mass bands were observed (Fig. 1B).

**Steady-state ATPase and Actin Binding Activity of HuM3MD**—The steady-state ATPase activity as a function of the actin concentration was measured for HuM3MD (Fig. 2A, Table 1). The basal ATPase activity (0.05 s\(^{-1}\)) was markedly activated by actin. The maximum rate of ATPase activity (\(V_{max}\)) is 0.54 s\(^{-1}\) with \(K_{act}\) of 0.13 \(\mu M\). The ATP concentration at half-maximum of the steady-state ATPase rate (\(K_{ATP}\)) is ~7-fold increased by actin. The \(K_{ATP}\) is 8.0 and 53.0 \(\mu M\) in the absence and presence of saturated actin, respectively (Table 1). The addition of exogenous CaM did not influence the actin-activated ATPase activity, consistent with the construct with no IQ motifs. The time course of Pi liberation by acto-HuM3MD was linear within the reaction time (30 min) without the ATP regeneration system, in contrast to myosin V (26).

The steady-state actin binding activity of HuM3MD was examined by the actin co-sedimentation assay in the presence of 1 mM ATP and the ATP regeneration system. The amount of actin-co-precipitated HuM3MD was saturated at a low concentration of actin in the presence of ATP. Fig. 2B shows the fraction of bound HuM3MD plotted against total actin concentration. Based upon Equation 1, a fraction of bound HuM3MD follows the parabolic saturation curve against total actin concentration. Based upon the obtained results, the affinity of HuM3MD to actin was estimated to be 0.15 \(\mu M\). The value agrees well with the \(K_{act}\) value of the actin-activated ATPase activity. These results suggest that HuM3MD keeps attaching to actin with high affinity during the ATPase cycle.

**Actin Binding to HuM3MD**—The rate of actin binding to HuM3MD was measured using pyrene-actin. The fluorescence intensity of pyrene-actin was rapidly decreased upon the addition of HuM3MD as a result of the binding of HuM3MD to pyrene-actin, as is known for other myosins (27–34). HuM3MD was mixed with pyrene-actin in the presence and absence of ADP, and the time course of the change in the fluorescence intensity was monitored (Fig. 3A, inset). The time
Kinetic Mechanism of Human Myosin III

A, the steady-state ATPase activity of acto-HuM3MD as a function of actin concentration. The ATPase activity of acto-HuM3MD was measured in the presence of 0.5 mM ATP, 0.01 mM myosin head and various concentrations of actin above 0.05 mM were used. A solid line is calculated based on the equation, \( v = \left[ V_{\text{max}} - V_{\text{o}} \right]/[\text{actin}] + k_{\text{actin}} \cdot \langle [\text{actin}] \rangle + V_{\text{o}} \). According to the analysis, the basal ATPase activity \( (V_o) \) was obtained for 0.05 s\(^{-1}\). The maximum ATPase activity at saturating actin concentration \( V_{\text{max}} \) was 0.54 s\(^{-1}\), with \( K_{\text{actin}} \) of 0.13 mM. The error bars indicate S.E. for \( n = 3 \) from three independent preparations.

B, steady-state binding of HuM3MD to actin. An actin co-sedimentation assay of HuM3MD was performed in the presence of 0.5 mM ATP, 1 mM ATP, ATP regeneration system, and various concentrations of actin. The pellet and supernatant were analyzed by SDS-PAGE (inset) as described under "Experimental Procedures." The fraction of bound HuM3MD with actin was plotted as a function of actin concentration. The solid line is the best fit to a hyperbolic curve according to Equation 1 with \( K_d \) of 0.15 ± 0.05 mM.

TABLE 1

Steady-state ATPase activity of HuM3MD

|        | \( V_{\text{max}} \) | \( K_{\text{ATP}} \) | \( K_{\text{actin}} \) |
|--------|----------------------|---------------------|----------------------|
| Without actin | 0.05 ± 0.002 | 8.0 ± 0.4 |                    |
| With actin   | 0.54 ± 0.02 | 53.0 ± 2.4 | 0.13 ± 0.03 |

-course of the change in fluorescence intensity followed a single exponential, and the observed apparent rate constants at various actin concentrations were plotted (Fig. 3A). The binding rate constant increased linearly with actin concentration to yield the second order rate constant \( (k_{-o}) \) of 29.8 \( \mu M^{-1} s^{-1} \). The dissociation rate constant \( (k_{+a}) \) was estimated from the y intercept to be 2.3 s\(^{-1}\). The actin binding rate constant and the dissociation constant were not significantly affected by ADP binding (Fig. 3A).

The dissociation of HuM3MD from pyrene-actin was measured by monitoring the increase in the fluorescence intensity upon the addition of unlabeled actin filament in the presence and absence of ADP (Fig. 3B). The change in fluorescence was followed by two exponential kinetics. The value of the fast phase agrees well with the one obtained from the binding assay (Fig. 3A). Therefore, we assessed \( k_{-a} = 2.05 s^{-1} \), and \( k_{+a} = 1.7 s^{-1} \) (Table 2). The dissociation constant for the actin binding was thus calculated to be \( K_d = 68.8 \text{nM} \) in the absence of nucleotide and \( K_{10} = 71.4 \text{nM} \) in the presence of ADP.

MgATP Binding to HuM3MD and Acto-HuM3MD—The rate of ATP binding to HuM3MD was measured by using the fluorescent nucleotide mantATP (Fig. 4A). The increase in
the fluorescence intensity of mantATP upon binding to HuM3MD followed a single exponential (Fig. 4A, inset), and the observed rates are linearly increased with mantATP concentration to yield the second order rate constant for mantATP binding ($k_2$) of 0.14 μM$^{-1}$ s$^{-1}$. The extrapolation of the straight line intercepting the y axis at 0.2 s$^{-1}$ in the absence of actin suggests the presence of the reverse reaction ($k_{-2}$).

The time course of mantATP binding to acto-HuM3MD was best fit to two exponentials (Fig. 4B, inset). The two exponentials are not due to the presence of mantATP isomers (35), because the two-exponential fluorescence changes were also observed using 2'-deoxy-mantATP having a single isomer. The amplitude of the fast phase is 60–70%, and the rates are linearly related to the mantATP concentration (Fig. 4B). The obtained second order rate constant determined from the slope ($k_2$') was 0.16 μM$^{-1}$ s$^{-1}$. The y intercept indicates the dissociation rate of mantATP from acto-HuM3MD ($k_{-2}$) to be 7.4 s$^{-1}$, suggesting that the ATP dissociation rate is significantly increased in the presence of actin. On the other hand, the second order rate constant of the slow phase was 0.03 μM$^{-1}$ s$^{-1}$. At present, we do not understand the origin of this slow change in the fluorescence intensity. A large rate constant of the reverse reaction is consistent with the high steady-state $K_{ATP}$ value (53 μM) (Table 1) in the presence of actin.

**MgATP-induced Dissociation of Acto-HuM3MD**—It is known that myosin quickly dissociates from actin upon the binding of ATP. The kinetics of ATP-induced dissociation of acto-HuM3MD was monitored by measuring the change in the light scattering of acto-HuM3MD after mixing the complex with ATP. The decrease in the light scattering due to the dissociation of HuM3MD from actin followed two exponentials (Fig. 4C, inset).
Kinetic Mechanism of Human Myosin III

FIGURE 5. ATP-induced dissociation of acto-HuM3MD. ATP-induced dissociation of acto-HuM3MD was measured by monitoring the decrease in the light scattering intensity. Dissociation rates as a function of ATP concentration are shown. The experiment was done in two different actin concentrations: 0.03 μM acto-HuM3MD (open circles) and 0.1 μM acto-HuM3MD (closed circles). All transients are fit to two exponentials, and the amplitude of the fast phase is 30–50%. The rates are plotted as a function of ATP concentration, and the data are fit to rectangular hyperbolas. The maximum values were 17.9 ± 0.4 s⁻¹ when 0.1 μM acto-HuM3MD was mixed with ATP (closed circles) and 11.4 ± 0.4 s⁻¹ when 0.03 μM acto-HuM3MD was mixed with ATP (open circles). The inset shows the time course of the light-scattering change after mixing 0.1 μM acto-HuM3MD with 600 μM ATP, k\text{FAST} = 14.9 s⁻¹ and k\text{SLOW} = 1.8 s⁻¹. AU, arbitrary units.

5, inset). The rates of the fast phase are dependent hyperbolically on ATP concentration (Fig. 5). The experiment was done with two different actin concentrations. When acto-HuM3MD (0.03 μM actin) was mixed with ATP, the maximum rate was 11.4 s⁻¹. This value is significantly lower than the rate of ATP binding, suggesting that the maximum value represents the actin dissociation step (kₐ₋ₐ + kₐ₋ₐ(actin)). The observed maximum rate constant was significantly increased at higher actin concentration (0.1 μM) to 17.9 s⁻¹. Hence, the second order rate constant for the association step (kₐ₋ₐ) was determined to be 92.9 μM⁻¹ s⁻¹. Accordingly, the rate constant for the dissociation acto–HuM3MD (kₐ₋ₐ) was calculated to be 8.6 s⁻¹. Based upon these values, 0.09 μM was obtained for the dissociation constant of acto–HuM3MD-ATP (Kₐ). This value is in good agreement with the Kₐ determined by the steady-state ATPase and actin binding experiments (Fig. 2). The association rate constant for ATP binding to acto–HuM3MD determined from initial slope (Kₐ’Kₐ₋ₐ) was 0.14 ± 0.01 μM⁻¹ s⁻¹, which is consistent with that obtained by measuring directly using mannitol (Fig. 4). The rates of the slow exponential phase were also hyperbolically related to ATP concentration. It could be due to the presence of a different conformer of AM species. But we do not have enough evidence to justify the presence of such a conformer.

Quench Flow Measurements of ATP Hydrolysis by HuM3MD and Acto-HuM3MD—Fig. 6 shows the time course of Pᵢ release of a HuM3MD ATPase reaction measured by a quenched flow experiment. Both in the absence and presence of actin, the time course of Pᵢ release showed an initial rapid phase followed by a slow linear phase. It has been known that the initial rapid Pᵢ release phase (Pᵢ-burst) is due to the formation of myosin·ADP·Pᵢ complex, in which the bound Pᵢ is released by acid quench. The observed Pᵢ-burst size was 0.63 mol/mol, suggesting that a significant fraction of HuM3MD is in prehydrolysis form (M·ATP). The ATP binding rate at the given ATP concentration was 2.8 s⁻¹, and the rate of reverse reaction was 0.2 s⁻¹ (Fig. 4), indicating that ~7% of HuM3MD is present in the ATP-unbound form. Based upon these numbers, the equilibrium constant of the hydrolysis step (Kₐ) was estimated to be 2.1. The observed steady-state rate of Fig. 6A was consistent with that determined by the conventional method (Fig. 2, Table 1). The observed rate constant of the initial phase was 0.14 s⁻¹. This value was rectified by using the rate constants of the ATP binding step at a given ATP concentration to yield 0.15 s⁻¹ for the hydrolysis step (kₐ₋ₐ + k₋ₐ). Based upon the equilibrium constant of the hydrolysis step (Kₐ) estimated above, kₐ₋ₐ and k₋ₐ were estimated to be 0.1 and 0.05 s⁻¹, respectively. The rate of ATP hydrolysis step in the absence of actin was quite slow compared with other myosins, such as myosin II, myosin V, and myosin VI, and predominantly explains the steady-state ATPase turnover rate.
On the other hand, the initial ATP hydrolysis rate was markedly increased in the presence of actin (Fig. 6B). The observed P_i-burst size was 0.19 mol/mol. Based upon the result of Fig. 4, the rate constants of ATP binding to and ATP dissociation from acto-HuM3MD in the assay conditions are 3.2 and 7.4 s^{-1}, respectively. Accordingly, ~70% of HuM3MD is the nucleotide-unbound form in the assay conditions. In the presence of saturated ATP concentration, the P_i-burst size of 0.63 mol/mol is expected. The equilibrium of the ATP hydrolysis step can be determined by the fraction of the fast P_i-burst phase and the calculated values of \( k_{-3}' \) and \( k_{-3} \) are 1.7, 2.7, and 1.6 s^{-1}, respectively. The slope of the slow linear phase is 0.1 s^{-1}, which is similar to the value obtained in the ATPase assay under comparable conditions.

The rate constant of the ATP hydrolysis step of the actin-bound form of HuM3MD was significantly larger than the \( V_{max} \) of the actin-activated ATPase activity (Fig. 2). Therefore, the ATP hydrolysis step of acto-HuM3MD does not determine the overall ATPase cycle rate, although this step partially contributes to the cycle rate. It should be noted that the hydrolysis rate is much higher for the actin-bound form of HuM3MD. This is quite different from any other myosin, including conventional myosin, in which the ATP hydrolysis rate is significantly decreased with bound actin and the ATP hydrolysis predominantly takes place in the actin-dissociated form (36), although the overall ATPase cycle rate is largely accelerated due to 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 III ATPase reaction.

**Kinetics of ADP Binding to and Dissociation from HuM3MD and Acto-HuM3MD**—The rate of ADP binding to HuM3MD and acto-HuM3MD was measured by monitoring the change in the fluorescence intensity of mantADP upon binding. Both in the presence and absence of actin, the fluorescence intensity of mantADP increased after mixing with HuM3MD, and the time course of increase in fluorescence intensity followed a single exponential (Fig. 7A, inset). The observed rates were linearly increased with mantADP concentration in both the absence and presence of actin (Fig. 7A). In the absence of actin, the second order rate constant for mantADP binding to HuM3MD (\( k_{-3} \)) was 0.40 \( \mu \)M^{-1} s^{-1} (Fig. 7A). The y intercept of the mantADP dependence gave the mantADP off rate constant (\( k_{+3} \)) of 6.5 s^{-1}. In the presence of actin, the second order rate constant for mantADP binding (\( k'_{-3} \)) was determined to be 0.58 \( \mu \)M^{-1} s^{-1}. The mantADP dissociation rate constant (\( k'_{+3} \)) of 6.5 s^{-1} was obtained from the y intercept.

The rate of ADP dissociation was determined by measuring the decrease in the fluorescence intensity of mantADP bound to HuM3MD upon the addition of 1 mM ATP (Fig. 7B). Based upon the result of ATP binding step determined in Fig. 4, the rate of ATP binding to HuM3MD at 1 mM ATP is >100 s^{-1}; therefore, the observed rate of the decrease in the fluorescence intensity represents the ADP off rate from HuM3MD. The time course of the change in fluorescence intensity followed a single exponential to yield the rate constant of 6.6 and 6.9 s^{-1} in the absence and presence of actin, respectively. These values agree well with the ADP dissociation rate constants estimated in Fig. 7A.

**The Effect of Phosphorylation of HuM3MD on Its Motor Function**—We previously reported that the construct that contains the kinase domain, the motor domain, and the IQ motif shows a high \( K_{actin} \) value (112 \( \mu \)M) (8). This value is quite different from that obtained in the present study using HuM3MD (Fig. 2A). Since HuM3MD does not contain the kinase domain, the purified protein is expected to be unphosphorylated. On the other hand, the construct used in the previous study is likely to be autophosphorylated during the ATPase assay. To examine the effect of phosphorylation of the motor domain of myosin III
on its ATPase activity, we measured the ATPase activity of HuM3MD in the presence of M3KD (Fig. 8, Table 3). Phosphorylation of HuM3MD by M3KD was saturated at 60 min (not shown); therefore, we monitored the ATPase activity 1 h after the addition of ATP. To avoid the inhibition of ATPase by liberated ADP, the ATPase activity was measured in the presence of an ATP-regeneration system. The basal ATPase activity and the $V_{\text{max}}$ of the actin-activated ATPase activity were 0.065 and 0.64 s$^{-1}$, respectively. The values are similar to those of dephosphorylated HuM3MD (Fig. 2A). On the other hand, $K_{\text{actin}}$ was ~500-fold increased in the presence of M3KD, and the value is consistent with the high $K_{\text{actin}}$ value obtained in the previous work (8). The result suggests that autophosphorylation of HuM3MD regulates the affinity of HuM3MD to actin filaments during the ATPase cycle.

**DISCUSSION**

In the present study, we clarified the mechanism of actomyosin IIIA motor function by analyzing the ATP hydrolysis cycle, which is closely coupled with the cross-bridge cycling of actomyosin (Scheme 1). Several key findings were made that suggest a unique actin-activated ATP hydrolysis mechanism of human myosin IIIA. First, the ATP hydrolysis step of myosin IIIA is quite slow, but actin markedly accelerates the ATP hydrolysis rate. Second, the affinity of myosin IIIA-ATP for actin is much higher than the conventional “weak actin binding” state. Because of these unique features, it is thought that myosin IIIA predominantly hydrolyzes ATP via the actin-attached pathway. On the other hand, the ADP off rate is much higher than the entire ATP hydrolysis cycle rate; thus, it is not rate-limiting. These results suggest that myosin IIIA is not a conventional high duty ratio motor; however, myosin IIIA may travel on actin filaments for a certain distance before completely dissociating from actin because of the unusually high affinity of myosin IIIA-ATP for actin.

**Acceleration of the ATP Hydrolysis Step by Actin**—One of the most interesting findings is that the $P_f$ burst rate (ATP hydrolysis rate) of the actin-dissociated form of myosin IIIA is quite low, and the rate is comparable with the basal ATPase cycle rate. It was originally found for skeletal myosin II that the myosin ATPase reaction shows the initial rapid $P_f$ release followed by a slow steady-state $P_f$ liberation (37). This is because myosin rapidly hydrolyzes ATP to form a M-ADP-P$_i$ ternary complex, which is labile by acid quench to release the bound $P_f$. Subsequently, it is found that this is common to other myosins (27–30, 33, 34). Because myosin is rapidly dissociated from actin upon ATP binding and hydrolyzes ATP, it has been thought that ATP hydrolysis takes place in actin-dissociated myosin, and this is not only for conventional myosin II but also myosin V and myosin VI that have a high duty ratio and show processive movement on actin (28, 29). On the other hand, the present study revealed that the ATP hydrolysis rate of myosin IIIA of the actin-dissociated form is much slower than the actin-activated ATPase cycle rate, indicating that it is unlikely that the actin-dependent ATPase cycle goes through the actin-dissociated form of myosin IIIA.

Consistent with this notion, the $P_f$ burst rate was much higher (30-fold) in the presence of actin that can explain the overall ATPase cycle rate. Quite recently, we reported that the ATP hydrolysis rate of myosin IXb is slow and determines the overall ATPase cycle rate (38). Furthermore, it was found for myosin VIIA that the actin-attached form of myosin hydrolyzes ATP much faster than the actin-dissociated form (34). Present results together with these previous findings suggest that the binding of actin accelerates ATP hydrolysis for certain types of myosin. It has been thought that the activation of the ATPase cycle of myosin by actin is due to the marked increase in the product release rate, especially the $P_f$ off rate, which is due to the opening of the “back door” by actin binding (39, 40). However, our results suggest that actin binding also changes the relative location of the phosphate moiety of ATP and the catalytic water (41), thus accelerating the hydrolysis reaction. It is plausible that such a movement induced by actin binding may be common among various myosins, but it may result in the opposite outcome (36).

Another characteristic of actomyosin IIIA ATPase reaction is the extremely low $K_{\text{actin}}$ value. The low $K_{\text{actin}}$ value was also found for myosin Va, and this is predominantly due to the low $K_a$ value (28, 42). On the other hand, the low $K_a$ value (M-ATP $\rightleftarrows$ AM-ATP) is critical to explain the low $K_{\text{actin}}$ in the case of the actomyosin III ATPase reaction. This is due to the extremely slow dissociation rate of myosin IIIA from actin ($k_{-d}$) and the large second order rate constant of the binding of myosin IIIA-ATP to actin ($k_{+d}$).

Based upon the above finding, the following scenario can be proposed for the actomyosin IIIA ATPase reaction. The actin-attached ATP hydrolysis rate is much faster than that of the
actin-dissociated form. Because AM-ATP – M:ATP is in fast equilibrium, myosin IIIA dissociated from actin upon ATP binding and quickly reassociated with actin (AM-ATP), and the bound ATP was hydrolyzed.

The Kinetic Mechanism of Human Myosin III ATPase Cycle—Using the experimentally determined kinetic constants of elementary steps, the overall ATP hydrolysis cycle pathway was analyzed by using computer simulation. All rate constants and equilibrium constants obtained in the present study are summarized in Table 2.

As described above, the ATP hydrolysis rate of human myosin IIIA in the absence of actin was very slow and partially explained the basal ATPase cycle rate. The rate of phosphate release from HuM3MD-ADP-P$_i$ ($k_{o,4}$) could not be measured because of slow hydrolysis. The $k_{o,4}$ value is calculated by computer simulation, and $k_{o,4} = 0.14 - 0.16 \text{ s}^{-1}$ can explain the basal ATPase activity of HuM3MD ($0.05 \pm 0.02 \text{ s}^{-1}$). Based upon the simulation, M-ATP (66%) and M-ADP-P$_i$ (33%) are the predominant steady-state intermediates.

The ATPase cycle rate of human myosin IIIA was increased by actin to 10-fold. The ATP hydrolysis step is significantly enhanced (30-fold) by actin as described above, and it is thought that ATP hydrolysis takes place in the actin-associated form of myosin IIIA. The rate of the formation of AM-ADP (66%) and AM-Pi (33%) is 1.14–1.26 s$^{-1}$ (30-fold) by actin as described above, and it is thought that ATP hydrolysis takes place in the actin-associated form of myosin IIIA.

Although $K_a$ could not be experimentally determined, the $K_a$ value of $0.1 - 0.3 \mu M$ was obtained by computer simulation based upon the rate constants determined in the present study. Based on the kinetic parameters determined, we calculated the steady-state distribution of intermediates during the actomyosin IIIA ATP hydrolysis cycle under the physiological ATP concentration (2 mM) (Fig. 9A). The AM-ATP and AM-ADP-P$_i$ are the predominant steady-state intermediates, and each intermediate populates 45%. Based upon these kinetic parameters, the actin-activated ATPase activity was calculated as a function of actin concentration (Fig. 9B). The calculated values agree with those of experimentally determined values over a wide range of actin concentrations, supporting the validity of the kinetic model shown in Fig. 9A.

It was shown previously that the steady-state ATPase activity is decreased in the presence of ADP. For instance, 0.4 mM ADP inhibited 50% of the activity measured in the presence of 0.2 mM ATP (8). The inhibition of the ATPase activity by ADP was well explained based upon the obtained kinetic parameters in the present study, and 45% inhibition by ADP was obtained under the above conditions. The inhibition is primarily due to the fast ADP binding to myosin IIIA.

Is Myosin IIIA a Processive Motor?—The function of motor proteins can be classified into two types (i.e. force producer and cargo transporter). The former type of myosin spends a majority of the cross-bridge cycle time with the actin-dissociated form. This enables multiple myosin molecules to interact with a single actin filament, thus producing a large force. On the other hand, the latter type of myosin walks on actin filament without dissociation from actin. A typical one is myosin V, a two-headed myosin, in which each head spends a majority of the cycle time on actin with highly coordinated binding of the two heads to actin. The former and latter types of motors are often referred to as nonprocessive and processive motors, respectively. Because myosin IIIA is thought to be a single-headed myosin, it is unlikely to move on actin like myosin V. On the other hand, it has been suggested that myosin IX, having a single-headed structure, moves processively on actin filaments using a multimolecule in vitro actin gliding assay (43, 44). Quite recently it was shown, using two different single molecule techniques, that myosin IX moves processively on actin filaments (45). These findings indicate that a single-headed myosin can move processively on actin filaments. Interestingly, recent kinetic analysis of the actomyosin IX ATPase reaction revealed that myosin IX is not a classical high duty ratio motor (38, 46). Unique features of myosin IX are that it has a slow ATP hydrolysis step and a high affinity for actin in the presence of ATP (38, 46). It spends a majority of ATP hydrolysis cycling time with the “weak actin binding” form, M-ATP. A critical finding was that M-ATP and M-ADP-P$_i$ have very high affinity for actin, unlike...
Kinetic Mechanism of Human Myosin III

other characterized myosins (38). Actually, the dissociation rate of the ATP-bound form of myosin IX from actin is quite slow, and it was proposed that myosin IX moves to the next binding site on actin filaments before diffusing away from the actin filament, presumably through the actin-tethering sites (38). There are remarkable similarities of the kinetic characteristics between myosin IX and myosin IIIA. First, the affinity of the ATP-bound form for actin is quite high for both myosins. Second, the rate of the ATP hydrolysis step of these myosins in the actin-dissociated form is very low. Because of these properties, ATP hydrolysis of these myosins takes place with the actin-associated form. Therefore, it is plausible that myosin IIIA may move processively on actin filaments using a similar mechanism as myosin IX.

Although the affinity of M-ATP for actin is high and actin binding accelerates the ATP hydrolysis, we think that the hydrolysis step does not form tight mechanical coupling between the actin and myosin interface. In other words, the interaction of myosin with actin promotes the hydrolysis, but the interaction in this step does not produce power stroke or reverse power stroke.

For myosin IX, a large insertion near the loop 2 region is suggested to play a role as an actin-tethering site. On the other hand, it was reported that the C-terminal end of the tail domain of myosin IIIA has an affinity for actin (7). This region may function as an actin-tethering site to support the continuous movement of myosin IIIA on actin filaments. Further studies are required for clarifying the mechanism of movement of myosin III on actin filaments.

Autophosphorylation of Myosin IIIA—The present results indicate that the phosphorylation of myosin IIIA markedly decreases the affinity for actin in the presence of ATP without changing the $V_{\text{max}}$. Based upon our kinetic model, we assigned that the phosphorylation alters $K_{\text{d}}$ and/or $K_{\text{a}}$ on the ATPase cycle. This means that myosin IIIA spends a significant of time during the ATP hydrolysis cycle in the actin-dissociated form. Although the role of autophosphorylation on the mechanical function of myosin IIIA is obscure at present, our results raise an idea that the phosphorylation abolishes the continuous movement of myosin IIIA on actin filaments. Supporting this notion, it has been shown that the kinase deletion mutant of fish myosin IIIA shows more extensive localization at the tip of filopodia of HeLa cells, whereas the full-length myosin IIIA localized throughout the cytoplasm without significant localization at the tip of filopodia (7). The results suggest that myosin IIIA without the kinase domain moves along the actin bundle in filopodia, whereas the one having the kinase domain does not. Further studies are required for clarifying the role of phosphorylation of myosin III on its motor function.

Acknowledgment—We are grateful to Kazuaki Homma (University of Massachusetts Medical School) for helpful discussion on the kinetic modeling.

REFERENCES

1. Montell, C., and Rubin, G. M. (1988) Cell 52, 757–772
2. Dose, A. C., and Burnside, B. (2000) Genomics 67, 333–342
3. Dose, A. C., and Burnside, B. (2002) Genomics 79, 621–624
4. Dose, A. C., Hillman, D. W., Wong, C., Sohler, L., Lin-Jones, J., and Burnside, B. (2003) Mol. Biol. Cell 14, 1058–1073
5. Battelle, B. A., Andrews, A. W., Calman, B. G., Sellers, J. R., Greenberg, R. M., and Smith, W. C. (1998) J. Neurosci. 18, 4548–4559
6. Walsh, T., Walsh, V., Vreugde, S., Hertzano, R., Shahin, H., Haika, S., Lee, M. K., Kanaan, M., King, M. C., and Avraham, K. B. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7518–7523
7. Les Erickson, F., Corsa, A. C., Dose, A. C., and Burnside, B. (2003) J. Biol. Chem. 278, 21352–21360
8. Komaba, S., Inoue, A., Maruta, S., Hosoya, H., and Ikebe, M. (2003) J. Biol. Chem. 278, 116, 683–693
9. Sokolov, M., Lyubarsky, A. L., Strissel, K. J., Savchenko, A. B., Govardovskii, V. I., Pugh, E. N., Jr., and Arshavsky, V. Y. (2002) Neuron 34, 95–106
10. Kouloff, M., Elia, N., Joel-Almagor, T., Timberg, R., Zars, T. D., Hyde, D. R., Minke, B., and Selinger, Z. (2003) EMBO J. 22, 459–468
11. Cronin, M. A., Diao, F., and Tsunoda, S. (2004) J. Cell Sci. 117, 4797–4806
12. Smith, L., Su, X., Kang, L. W., Amzel, L. M., and Montell, C. (2003) Neuron 39, 121–132
13. Peterson, J. J., Tam, B. M., Moritz, O. L., Shalermer, C. L., Dugger, D. R., McDowell, J. H., Hargrave, P. A., Papamaster, D. S., and Smith, W. C. (2003) Exp. Eye Res. 76, 553–563
14. Ng, K. P., Kambara, T., Matsuura, M., Burke, M., and Ikebe, M. (1996) Biochemistry 35, 9392–9399
15. Lin-Jones, J., Parker, E., Wu, M., Dose, A., and Burnside, B. (2004) J. Cell Sci. 117, 5825–5834
16. Smith, L., Su, X., Lin, P., Zhi, G., and Stull, J. T. (1999) J. Biol. Chem. 274, 29433–29438
17. Thorn, K. S., Ubersax, J. A., and Vale, R. D. (2000) J. Cell Biol. 151, 1093–1100
18. Spudich, J. A., and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871
19. Kouyama, T., and Mihashi, K. (1981) J. Cell Sci. 47, 121–132
20. Smith, L., Su, X., Lin, P., Zhi, G., and Stull, J. T. (1999) J. Biol. Chem. 274, 29433–29438
21. El Mezgueldi, M., Tang, N., Rosenfeld, S. S., and Ostap, E. M. (2002) J. Biol. Chem. 277, 21514–21521
22. De La Cruz, E. M., Wells, A. L., Rosenfeld, S. S., Ostap, E. M., and Sweeney, H. L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13726–13731
23. De La Cruz, E. M., Ostap, E. M., and Sweeney, H. L. (2001) J. Biol. Chem. 276, 32373–32381
24. Kovacs, M., Wang, F., and Sellers, J. R. (2005) J. Biol. Chem. 280, 15071–15083
25. Yang, Y., Kovacs, M., Xu, Q., Anderson, J. B., and Sellers, J. R. (2005) J. Biol. Chem. 280, 32061–32068
26. Henn, A., and De La Cruz, E. M. (2005) J. Biol. Chem. 280, 39665–39676
27. Homma, K., and Ikebe, M. (2005) J. Biol. Chem. 280, 29381–29391
28. Watanabe, S., Ikebe, R., and Ikebe, M. (2006) J. Biol. Chem. 281, 7151–7160
29. Woodward, S. K., Eccleston, J. F., and Geeves, M. A. (1991) Biochemistry 30, 422–430
30. White, H. D., Belknap, B., and Webb, M. R. (1997) Biochemistry 36, 11828–11836
31. Kanazawa, T., and Tomonura, Y. (1965) J. Biochem. (Tokyo) 57, 604–615
32. Kambara, T., and Ikebe, M. (2006) J. Biol. Chem. 281, 4949–4957
33. Yount, R. G., Lawson, D., and Raymont, I. (1995) Biochem. J. 318, 445–495
34. Pate, E., Naber, N., Matuska, M., Franks-Skiba, K., and Cooke, R. (1997) EMBO J. 16, 4797–4806
35. Stein, J., and Montell, C. (2004) J. Cell Biol. 165, 599–608
36. Kanaan, M., King, M. C., and Avraham, K. B. (2002) Mol. Biol. Cell 13, 11828–11836
37. Kanazawa, T., and Tomonura, Y. (1965) J. Biochem. (Tokyo) 57, 604–615
38. Kanaan, M., King, M. C., and Avraham, K. B. (2002) Mol. Biol. Cell 13, 11828–11836
39. Yount, R. G., Lawson, D., and Raymont, I. (1995) Biochem. J. 318, 445–495
Kinetic Mechanism of Human Myosin III

Biochemistry 36, 12155–12166
41. Fisher, A. J., Smith, C. A., Thoden, J. B., Smith, R., Sutoh, K., Holden, H. M., and Rayment, I. (1995) Biochemistry 34, 8960–8972
42. Yengo, C. M., and Sweeney, H. L. (2004) Biochemistry 43, 2605–2612
43. Post, P. L., Tyska, M. J., O’Connell, C. B., Johung, K., Hayward, A., and Mooseker, M. S. (2002) J. Biol. Chem. 277, 11679–11683
44. Inoue, A., Saito, J., Ikebe, R., and Ikebe, M. (2002) Nat. Cell Biol. 4, 302–306
45. Nishikawa, M., Nishikawa, S., Inoue, A., Iwane, A. H., Yanagida, T., and Ikebe, M. (2006) Biochem. Biophys. Res. Commun., 343, 1159–1164
46. Nalavadi, V., Nyitrai, M., Bertolini, C., Adamek, N., Geeves, M. A., and Bahler, M. (2005) J. Biol. Chem. 280, 38957–38968