Kinetic Mechanism of the Fastest Motor Protein, Chara Myosin

Received for publication, December 26, 2006, and in revised form, May 4, 2007. Published, JBC Papers in Press, May 7, 2007, DOI 10.1074/jbc.M611802200

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Chara corallina class XI myosin is by far the fastest molecular motor. To investigate the molecular mechanism of this fast movement, we performed a kinetic analysis of a recombinant motor domain of Chara myosin. We estimated the time spent in the strongly bound state with actin by measuring rate constants of ADP dissociation from actin-motor domain complex and ATP-induced dissociation of the motor domain from actin. The rate constant of ADP dissociation from acto-motor domain was >2800 s⁻¹, and the rate constant of ATP-induced dissociation of the motor domain from actin at physiological ATP concentration was 2200 s⁻¹. From these data, the time spent in the strongly bound state with actin was estimated to be <0.82 ms. This value is the shortest among known values for various myosins and yields the duty ratio of <0.3 with a Vₘₐₓ value of the actin-activated ATPase activity of 390 s⁻¹. The addition of the long neck domain of myosin Va to the Chara motor domain largely increased the velocity of the motility without increasing the ATP hydrolysis cycle rate, consistent with the swinging lever model. In addition, this study reveals some striking kinetic features of Chara myosin that are suited for the fast movement: a dramatic acceleration of ADP release by actin (1000-fold) and extremely fast ATP binding rate.

Myosin is an actin-based motor protein that converts chemical energy liberated by the ATP hydrolysis into directed movement of actin filaments. Phylogenetic analysis of myosin sequences revealed that there are at least 24 classes of myosin (1). Motor functions, such as motility and ATP hydrolysis activity, vary significantly among these classes of myosin. Among those known so far, myosin from alga Chara corallina is the fastest, moving actin filaments at 40–60 μm/s in the in vitro motility assay (2–4). This velocity is ~10 times faster than that of the fast skeletal muscle myosin (5). We have been interested in the mechanism how this plant myosin can move so fast. To understand the molecular mechanism of the fast movement, detailed kinetic analysis of Chara myosin is required. However, the yield of native Chara myosin was very low. In addition, the activity of Chara myosin was impaired after multiple purification steps, so that in vitro actin sliding velocity of Chara myosin purified to homogeneity was only one-fourth to one-half of that measured with crude extracts of Chara cells. Therefore, it has been difficult to measure the detailed kinetics of native Chara myosin (2). Thus, expression and purification of functional recombinant Chara myosin is crucial to obtain reliable biochemical information. The cDNA of the Chara myosin heavy chain has been cloned (6, 7). The sequence predicts that the myosin consists of a motor domain that belongs to class XI myosin, a neck domain comprising six tandem repeats of IQ motifs and serving as binding sites for six light chains, an α-helical coiled-coil domain supporting dimer formation, and a globular tail domain (see Fig. 1A, native Chara myosin). The functional expression of myosins requires co-expression of the light and heavy chains. Co-expressed calmodulin was bound to IQ motifs and functioned as light chains for some unconventional myosins or myosin fragments such as rat myosin I (myr-1) (8), human myosin IC (myr-3) (9), human myosin IIIA (10), chick myosin Va (11), mouse myosin Va (12–14), pig myosin VI (15), Drosophila myosin VIIB (16), mouse myosin VI (17), bovine myosin X (18, 19), and human myosin IXb (20). Sequence analyses of IQ motifs of these myosins using the Pfam protein families data base indicate that all the IQ motifs of these co-expressed myosins are typical calmodulin-binding IQ motifs (21); i.e. most of Pfam E-values are <10⁻³ and all are <10⁻¹ for IQ motifs of these co-expressed myosin fragments, whereas a Pfam E-value of <10⁻³ indicates significant comparison and an E-value in the range of 10⁻² to 10⁻¹ indicates a true relationship (22). In contrast, three IQ motifs of Chara myosin deviate from the typical calmodulin-binding IQ motif, i.e. Pfam E-values are 0.13, 0.092, and 4.8 for the first, fourth, and sixth IQ motifs, respectively. In accordance with this, Chara myosin heavy chain did not associate with Chara calmodulin when both polypeptides were co-expressed using the baculovirus-insect cell system. Because the light chains of Chara myosin are not well characterized, functional expression of Chara myosin heavy chain with light chains is now difficult. Recently, we succeeded in obtaining functional recombinant Chara myosin motor fragments by expressing the Chara myosin motor domain constructs without the light chain-binding region (23).

* This work was supported by Grants-in-Aid from the Ministry of Science, Culture and Education of Japan (to K. I. and K. Y.), by the Hamaguchi Foundation for the Advancement of Biochemistry (to K. I.), and by National Institutes of Health Grants AR048526, AR048898, and DC006103 (to M. I.). 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.

† The on-line version of this article (available at http://www.jbc.org) contains supplemental movie S1.

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In this study, we examined the kinetic properties of Chara myosin motor domain construct in detail (Fig. 1A, MD). This construct is similar to that reported previously (23) but does not have enhanced yellow fluorescent protein moiety because enhanced yellow fluorescent protein can interfere with fluorescence measurements in the stopped-flow apparatus. We made another Chara myosin motor domain construct, referred to as MD-Vneck (Fig. 1A, MD-Vneck). MD-Vneck consists of the Chara myosin motor domain and the neck and tail domains of mouse myosin Va. This construct was used to test whether the velocity of native Chara myosin can be restored using a lever arm with length and stiffness similar to native Chara myosin.

EXPERIMENTAL PROCEDURES

Protein Engineering and Expression—A baculovirus transfer vector for MD was generated as follows. Plasmid pTIKL Chara myosin MD (23) was mutated to remove enhanced yellow fluorescent protein and add a Myc-epitope sequence at the C-terminal end by site-directed mutagenesis using the ExSite PCR-based site-directed mutagenesis kit (Stratagene). The resultant construct, MD, contains residues 4–746 of the Chara myosin heavy chain, a flexible linker (GGG), a Myc-epitope sequence (EQKLISEEDL), and an His6 tag. This was subcloned into the baculovirus transfer vector pFastBac1 (Invitrogen) between the BamHI and HindIII sites.

A baculovirus transfer vector for MD-Vneck was generated as follows. Plasmid pTIKL Chara myosin MD was mutated to create the Ncol site, the Nhel site at the upstream region of the nucleotide sequence encoding residue 4 of the Chara myosin heavy chain, and the downstream region of the nucleotide sequence encoding residue 740 of the Chara myosin heavy chain, respectively. This plasmid was cut with Ncol and Nhel (an Ncol-Nhel fragment of Chara myosin MD). A baculovirus transfer vector for mouse myosin Va (M5Full) in pFastBac1 (24, 25) was mutated to create the Nhel site at the upstream region of the nucleotide sequence encoding residue 767 of the mouse myosin Va heavy chain. Because an Ncol site exists between an N-terminal tag and the N terminus of mouse myosin Va heavy chain, this plasmid was cut with Ncol and Nhel and ligated with the Ncol-Nhel fragment of Chara myosin MD. The resultant construct, MD-Vneck, encodes the N-terminal tag (MSYYH-HHHHHDYKDDDDKNIPTTENLYFQGQA) containing the sequence of His6 tag and FLAG tag (DYKDDDDK), residues 4–740 of Chara myosin heavy chain, linker consisting of two amino acids (AS), and residues 767–1853 of mouse myosin Va heavy chain. To express MD and MD-Vneck, an 800-ml culture of High Five™ cells (Invitrogen) was infected with viruses expressing the respective heavy-chain constructs. For MD-Vneck, mouse calmodulin was co-produced by coinfection with virus expressing mouse calmodulin (12). The infected cells were cultured in Erlenmeyer flasks (1 liter \times 4) at 28 °C and 130 rpm for 43 h.

The lever arm length of MD and MD-Vneck was determined using crystal structure of myosin Va (PDB code: 1W7J). Because the crystal structure of myosin Va has only one IQ motif, the length of six IQ motifs of MD-Vneck was calculated by multiplying the length of one IQ motif of the crystal of myosin Va by 6.

Protein Purification—MD was purified using the method of Homma et al. (12) with some modifications. Cells were harvested and washed with 150 mm NaCl, 10 mm Hepes, pH 7.4. The pelleted cells were suspended with 3 vol/g cells of buffer A (150 mm KCl, 5 mm MgCl2, 0.2 mm EGTA, 10 mm DTT, 10% glycerol, 30 mm Hepes, pH 7.4, and a mixture of protease inhibitors consisting of 0.1 mm phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 2 μg/ml pepstatin, 50 μg/ml N-p-tosyl-l-arginine methyl ester hydrochloride and 40 μg/ml 1-tosylamido-2-phenylethyl chloromethyl ketone) containing 1 mm ATP. Then 3 vol/g cells of buffer A containing 1 mm ATP, 2% Triton X-100 was added, and the ingredients were mixed. After incubation on ice for 15 min, the lysate was centrifuged at 228,000 × g for 30 min. The supernatant was incubated with 0.2 mg/ml F-actin, 50 mm glucose, and 20 units/ml hexokinase in a 50-ml tube on a rotating wheel for 2 h at 4 °C to hydrolyze completely residual ATP and centrifuged (228,000 × g for 30 min) to co-precipitate the MD. The pellets were washed once with buffer A and then resuspended with buffer B (300 mm KCl, 7 mm MgCl2, 10 mm β-mercaptoethanol, and 20 mm Hepes, pH 8.0) containing 5 mm ATP. The supernatant was mixed with 0.3 ml of nickel-nitritolotricarboxylic acid-agarose (Qiagen) in a 50-ml tube on a rotating wheel for 1 h at 4 °C. The resin suspension was then loaded on a column and was washed with 20 ml of buffer B containing 1 mm ATP and 10 mm imidazole, followed by 10 ml of buffer B containing 1 mm ATP and 15 mm imidazole and 10 ml of buffer B containing 10% glycerol and 15 mm imidazole. MD was eluted with buffer B containing 10% glycerol and 250 mm imidazole. The eluted MD was dialyzed overnight against 75 mm KCl, 4 mm MgCl2, 1 mm DTT, 10% glycerol, and 25 mm Hepes, pH 7.4, at 0–4 °C.

MD-Vneck was purified using the method of Li et al. (24) with some modifications. Cells were harvested and washed with 150 mm NaCl, 1 mm EGTA, and 10 mm Hepes, pH 7.4. The pelleted cells were suspended with 3 vol/g cells of buffer C (100 mm NaCl, 200 mm KCl, 10 mm MgCl2, 7 mm ATP, 5 mm EGTA, 2 mm DTT, 30 mm Hepes, pH 7.4, and a mixture of protease inhibitors). Then 3 vol/g cells of buffer C containing 2% Triton X-100 was added, and the ingredients were mixed. After incubation on ice for 15 min, the lysate was centrifuged at 228,000 × g for 30 min. The supernatant was mixed with 0.3 ml of anti-FLAG M2 affinity resin (Sigma) in a 50-ml tube on a rotating wheel for 3 h at 4 °C. The resin suspension was then loaded on a column and was washed with 30 ml of buffer D (300 mm KCl, 5 mm MgCl2, 1 mm ATP, 2 mm EGTA, 1 mm DTT, 30 mm Hepes, pH 7.4, and a mixture of protease inhibitors) containing 1 μM mouse calmodulin, followed by 3 ml of buffer D. MD was eluted with buffer D containing 0.2 mg/ml of 3×FLAG peptide (Sigma).

Mouse calmodulin was expressed in High Five™ cells by infecting virus expressing mouse calmodulin (12) and purified using the method of Awata et al. (26). Rabbit skeletal muscle actin was prepared using the method of Spudich and Watt (27). Protein concentration was determined using the Coomassie Plus protein assay reagent (Pierce) for MD and MD-Vneck with

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3 The abbreviations used are: MD, motor domain; S1, subfragment 1; DTT, dithiothreitol; mant, N-methylanthraniloyl.
Mechanism of Fast Chara Myosin Movement

Dictyostelium S1 as the standard. The concentrations of Dictyostelium S1 and actin were determined spectrophotometrically using extinction coefficients of 0.80 cm⁻²/mg at 280 nm for S1 (28) and 0.62 cm⁻²/mg at 290 nm for actin (29).

Western Blot Analysis—After SDS-PAGE, proteins in the gel were electrophotically transferred to nitrocellulose membrane. The membrane was incubated with blocking solution, 1% gelatin in TTBS (Tris-buffered saline, pH 7.5, containing 0.05% Tween 20), and reacted with mouse anti-calmodulin monoclonal antibody raised against a synthetic peptide equivalent to an amino acid sequence in the C terminus of all vertebrate calmodulins (Zymed Laboratories Inc., cat. no. 13-6900) (30). The protein band recognized by primary antibody was detected by alkaline phosphatase-conjugated goat anti-mouse IgG (Promega, cat. no. S3721).

ATPase and in Vitro Motility Assays—Steady-state ATPase activities were measured as described (23), except for that the reaction was done at 25 °C and assays of MD-Vneck were done in the presence of exogenous calmodulin (~1 μM). The velocity of MD was measured using an antibody-based version of the in vitro sliding filament assay (31), in which anti-c-Myc monoclonal antibody (Zymed Laboratories Inc., cat. no. 13-2500) was used. The velocity of MD-Vneck was measured using the standard in vitro motility assay (5), except that the glass surface was not coated with nitrocellulose, and exogenous calmodulin (~1 μM) was added in the assay buffer. Average sliding velocities were determined by measuring the displacements of actin filaments that were smoothly moving for distances greater than 10 μm.

Transient Kinetic Experiments—All kinetic experiments were done in 25 mM KCl, 4 mM MgCl₂, 1 mM DTT, and 25 mM Hepes, pH 7.4, using an Applied Photophysics SX18MV stopped-flow spectrophotometer (dead time: 1.15 ms). Measurements were done at 25 °C unless otherwise mentioned. Dissociation of acto-MD by ATP was monitored through changes in fluorescence intensities of pyrene-labeled actin stabilized by phalloidin. Pyrene-actin was excited at 365 nm, and the fluorescence was detected after passing through a 389 nm cut-off filter (32). Binding of mantATP and mantADP to MD was monitored by the increase of their fluorescence accompanying association of mantATP and mantADP with MD complex (33). Dissociation of mantADP from MD and acto-MD was monitored by the decrease of its fluorescence. mantATP and mantADP were excited at 290 nm via fluorescence resonance energy transfer from tryptophan of MD, and emission was observed after passing through a 389 nm cutoff filter.

RESULTS

Constructs, Expression, and Preparation

In this study, we designed and expressed two recombinant Chara myosin motor domain constructs. We refer to the first as MD and the second as MD-Vneck (Fig. 1A). MD contains only the Chara myosin motor domain and does not contain light-chain binding sites and light chains. The lever arm length of MD is 3 nm if the fulcrum of the converter rotation is assumed to be at the distal end of the SH1 helix (34). Because the primary structure deduced from the cDNA shows that Chara myosin has six light-chain binding IQ motifs (6), the lever arm length of native Chara myosin is expected to be the same as that of myosin Va, 24 nm (Fig. 1A). Thus the lever arm length of MD is one-eighth (3 nm/24 nm) that of native Chara myosin. MD-Vneck consists of the motor domain of Chara myosin and a neck, coiled-coil, and globular tail domains of mouse myosin Va. The lever arm length of MD-Vneck is 24 nm and is expected to be the same as that of native Chara myosin.

MD was expressed in insect High Five™ (Invitrogen). So far Sf9 cells have been used for the baculovirus-insect cell system to express myosins (8–20). However, High Five™ cells (Invitrogen) were used in this study, because the expression yields of Chara myosin constructs in these cells were more than two times larger than those in Sf9 cells. After expression in High Five™ cells, MD was purified by co-precipitation with actin and by nickel-affinity resin (Fig. 1B). The yield of MD was ~1 mg of protein from 2 × 10⁶ High Five™ cells (800 ml of culture). MD-Vneck was co-expressed with mouse calmodulin in High Five™ cells and purified with an anti-FLAG antibody affinity resin. The yield of MD-Vneck was ~0.1 mg of protein from 2 × 10⁶ High Five™ cells (800 ml of culture). Purified MD-Vneck contained two low molecular weight bands. We speculate that one of these bands is a post-translationally modified form of calmodulin that produced specifically in High Five™ cells, because both bands were recognized by anti-calmodulin monoclonal antibody raised against a synthetic peptide...
equivalent to an amino acid sequence in the C terminus of all vertebrate calmodulins (Fig. 1C) (30) and because only one band (lower molecular weight) was bound to MD-Vneck when co-expressed in Sf9 cells. In addition, no detectable bands were observed when only MD-Vneck was expressed in High Five™ cells. Anyway, both bands are most certainly calmodulin, because they showed calcium-dependent shift in mobility in SDS-PAGE (Fig. 1B). Judging from the molecular weight, the lower band seems to be a native calmodulin.

Steady-state ATPase Activity

The basal Mg$^{2+}$-ATPase activity of MD was 0.59 ± 0.045 phosphate release/s/head at 25 °C. In the presence of actin, the Mg$^{2+}$-ATPase activity of MD largely increased (Fig. 2). $V_{\text{max}}$ and $K_{\text{app}}$ values of MD were 390 ± 13 phosphate release/s/head and 16 ± 1.6 μM, respectively (25 °C). These values were similar to those of MD used in our previous study (23). $V_{\text{max}}$ and $K_{\text{app}}$ values of MD-Vneck were 220 ± 4.7 phosphate release/s/head and 10 ± 0.7 μM, respectively (25 °C). The same solution (25 mM KCl, 4 mM MgCl$_2$, and 25 mM Hepes, pH 7.4) was used for the steady-state ATPase activity measurement, motility assay, and 10° kinetic analysis, because kinetic analysis of single-headed MD is easier than double-headed MD-Vneck, and stopped-flow kinetics was plotted against ATP concentration (Fig. 3). This dissociation was monitored by the increase in the pyrene fluorescence (35). The mean $K_{\text{i}},{k_{\text{a}2}}$ value from four different MD preparations was 4.1 ± 0.1 μM$^{-1}$s$^{-1}$. The observed rate constant ($k_{\text{obs}}$) was plotted against low ATP concentrations (10–100 μM). The slope of the plot gave a value for $K_{\text{i}},{k_{\text{a}2}}$ of 4.1 μM$^{-1}$s$^{-1}$ in the example shown. The averaged $K_{\text{i}},{k_{\text{a}2}}$ value from four independent preparations of MD was 4.1 ± 0.1 μM$^{-1}$s$^{-1}$. Inset, 0.5 μM pyrene-acto-MD was mixed with 10 μM ATP (in final concentration). The increase in the fluorescence was fitted to a single exponential with a rate constant of 42 s$^{-1}$ (solid line). AU, arbitrary units.

Kinetic Analysis

ATP-induced Dissociation of Acto-MD—We used MD for kinetic analysis, because kinetic analysis of single-headed MD is easier than double-headed MD-Vneck, and stopped-flow measurements require a large quantity of protein. Kinetic modeling and simulations (11, 33) were performed by using a simplified reaction mechanism (Scheme 1). In all scheme and reactions in this work, A and M represent actin and MD, respectively. $k_{\text{a}1}$ and $k_{\text{a}2}$ are the forward and reverse rate constants in the presence of actin, respectively. $K_{\text{i}} = (k_{\text{a}2}/k_{\text{a}1})$ represents the equilibrium constant in the presence of actin. In the absence of actin, these are expressed as $k_{\text{a}1}, k_{\text{a}2}$, and $K_{\text{i}}$. The ATP-induced dissociation reaction of MD from actin can be described as a two-step process (Reaction 1), and the rate constant is expressed as $K_{\text{i}}k_{\text{a}2}[\text{ATP}]/(1 + K_{\text{i}}[\text{ATP}])$ (35).

\[
\begin{align*}
\text{AM} + \text{ATP} & \rightarrow \text{AM(}\text{ATP}\text{)} \\
\text{AM(}\text{ATP}\text{)} & \rightarrow A + M\cdot\text{ATP}
\end{align*}
\]

Reaction 1

The first step in Reaction 1 is the formation of a collision complex of acto-MD and ATP, which is in rapid equilibrium. The second step is dissociation of MD-ATP from actin following the isomerization of MD. When ATP concentration is low, the observed rate constant ($k_{\text{obs}}$) can be approximated as $K_{\text{i}},{k_{\text{a}2}}$[ATP]. To determine $K_{\text{i}},{k_{\text{a}2}}$, the acto-MD complex was dissociated by mixing with 10–100 μM ATP, and the dissociation rate was plotted against ATP concentration (Fig. 3). This dissociation was monitored by the increase in the pyrene fluorescence labeled on actin (36). The mean $K_{\text{i}},{k_{\text{a}2}}$ value from four different MD preparations was 4.1 ± 0.1 μM$^{-1}$s$^{-1}$.

To determine the $k_{\text{a}2}$ value, fitting the data to hyperbola at saturating ATP concentrations is needed (11, 14, 37, 38). However, the observed rate constant increased linearly with the increase in the ATP concentration and did not deflect from the linear line even until the detection limit of the stopped-flow apparatus (~1,000 s$^{-1}$). Thus we obtained $k_{\text{a}2}$ values at low temperatures (<13 °C), and the $k_{\text{a}2}$ value at 25 °C was estimated by extrapolating these values on an Arrhenius plot. Determination of a very high value in this way was used to estimate the $k_{\text{a}2}$ value for fast skeletal muscle myosin (35). The $k_{\text{a}2}$ values at 2.4, 5.3, 8.3, and 12.2 °C...
were 300, 430, 570, and 830 s⁻¹, respectively (Fig. 4A). The temperature dependence of $k_{+2}$ is shown in Fig. 4B. A linear Arrhenius plot was observed from 2.4 °C to 12.2 °C. The $k_{+2}$ value at 25 °C was estimated to be 2600 s⁻¹ by extrapolating this plot to 25 °C.

**ATP Binding to MD in the Absence of Actin**

ATP binding to MD in the absence of actin was measured by the enhancement of intrinsic tryptophan fluorescence and by the enhanced fluorescence of mantATP. Similar to several other myosin isoforms (38–41), tryptophan fluorescence of MD increased upon mixing with ATP (Fig. 5A, inset). The conserved tryptophan (Trp-510 in chicken skeletal muscle myosin, Trp-512 in chicken smooth muscle myosin, and Trp-501 in *Dictyostelium* myosin II) that contributes to the fluorescence enhancement upon binding to ATP (42) is present in *Chara* myosin (Trp-485). ATP-induced isomerization of MD in the absence of actin is thought to be a two-step process (Reaction 2), the formation of a collision complex of MD and ATP ($K_1$) followed by an almost irreversible isomerization of MD upon binding to ATP ($k_{+2}$). An asterisk in Reaction 2 indicates the enhancement of tryptophan fluorescence, which is caused by the isomerization.

When ATP concentration is low, the observed rate constant ($k_{obs}$) can be approximated as $K_1k_{+2}$ [ATP]. Thus the slope of a plot of $k_{obs}$ versus [ATP] at low ATP concentration gives an apparent second-order rate constant for ATP binding to MD ($K_1k_{+2}$) (Fig. 5A). The mean $K_1k_{+2}$ value from three independent preparations of MD was 9.8 ± 0.9 μM⁻¹ s⁻¹. This value is much higher than those of other myosins (Table 1).

At 25 °C, the observed rate constant increased linearly with increasing ATP concentrations (~100 μM) and did not deflect from the linear line even until the detection limit of the stopped-flow apparatus (~1000 s⁻¹). Thus we measured the rate constant at 3 °C and high ATP concentrations (Fig. 6). The observed rate constant showed a maximum value of 530 s⁻¹ at the ATP concentration range examined (up to 4 mM). The maximum rate of the fluorescence enhancement is thought to correspond to the rate of ATP hydrolysis ($k_{+3} + k_{-3}$, Reaction 3) (37, 38, 43).
Reaction 4, in which two asterisks show the enhanced fluorescence of mantATP (11, 33, 37, 44, 45).

\[
K_1 \quad M + \text{mantATP}^* \rightleftharpoons M(\text{mantATP}^*) \rightleftharpoons \text{MmantATP}^{**}
\]

**REACTION 4**

The mean \(k_{1,2}\) value obtained using mantATP from three independent preparations of MD was \(36 \pm 0.3 \, \mu M^{-1} \, s^{-1}\) (Fig. 5B), which is also much higher than those of other myosins (Table 1). The \(k_{1,2}\) value obtained using mantATP was more than three times larger than that obtained using the change in tryptophan fluorescence (Fig. 5A). The difference in \(k_{1,2}\) values between tryptophan fluorescence and mantATP was also observed in other myosins (37, 45). There are several possible reasons for this difference (see “Discussion”).

**ADP Dissociation from Acto-MD**

For myosins whose equilibrium dissociation constant of acto-MD for ADP (\(K_{ADP}\)) is low (<10 \(\mu M\)), the rate constant of ADP dissociation from acto-S1 (\(k_{ADP}\)) has been measured by observing the change of pyrene fluorescence labeled on actin or light scattering after mixing acto-myosin S1-ADP complex with an excess amount of ATP (11, 31, 46, 47). However, for myosins whose \(K_{ADP}\) is high (>100 \(\mu M\)), \(k_{ADP}\) has not been measured using pyrene or light scattering (48–51). The main reasons for this are that only a small fraction of acto-S1 is bound to ADP and most of the signal is due to acto-MD dissociation not accompanying ADP dissociation, even if ADP concentration is high (>100 \(\mu M\)). Likewise, pyrene fluorescence or light scattering could not be used to measure \(k_{ADP}\) of Chara MD because of the low affinity of acto-MD for ADP (\(K_{ADP}\) is 260 \(\mu M\), see below). Therefore, \(k_{ADP}\) was measured by observing the fluorescence decrease of mantADP accompanying the dissociation of mantADP from acto-MD-mantADP complex upon addition of excess ATP (Reaction 5 (9–11,14,17,38,45,52–54)).

**REACTION 5**

\[
\text{AM-mantADP}^{**} \quad \rightleftharpoons \text{AM} + \text{mantADP}^*
\]

Similar to \(k_{1,2}\), \(k_{ADP}\) was too fast to be measured at physiological temperatures (≈25 °C). Thus we obtained \(k_{ADP}\) values at low temperatures. The \(k_{ADP}\) values at 2.3, 5.0, 7.8, and 10.3 °C were 210 ± 16, 290 ± 14, 430 ± 25, and 590 ± 35 s⁻¹, respectively (Fig. 7A). The temperature dependence of \(k_{ADP}\) showed a linear Arrhenius plot (Fig. 7B). The \(k_{ADP}\) value at 25 °C was estimated to be 2800 s⁻¹, by extrapolating this plot to 25 °C.

**ADP Affinity for Acto-MD**—The equilibrium dissociation constant of acto-MD for ADP (\(K_{ADP}\)) was determined from the inhibitory effect of ADP on the ATP-induced acto-MD dissociation. ATP solution at a constant concentration (25 \(\mu M\)) was mixed with acto-MD equilibrated with various concentrations of ADP (final concentrations of 0 to 1500 \(\mu M\)). Because the active site of MD occupied by ADP cannot respond to ATP
(Reaction 6), the observed rate constant of ATP-induced acto-MD dissociation ($k_{o b s}$) decreases as the ADP concentration increases.

ADP from acto-MD stays in equilibrium at low concentrations of ATP, and the observed rate constant of acto-MD dissociation is defined by $K_k (k_{k +a}) [ADP]/(1 + [ADP]/K_{ADP})$ (37, 55). Hyperbolic fits to the $k_{o b s}$ versus [ADP] plots gave $K_{ADP}$ (Fig. 8). The mean $K_{ADP}$ value from four different MD preparations was 260 ± 39 μM. This value is double that for the fast skeletal muscle myosin (37), showing very low affinity of acto-MD for ADP (Table 1).

**ADP Binding to and Dissociation from MD in the Absence of Actin**—To investigate whether ADP release is accelerated by actin binding, we measured the ADP dissociation rate from MD in the absence of actin and compared it to that in the presence of actin. ADP dissociation from MD was followed by monitoring the decrease in fluorescence of mantADP upon the addition of excess ATP (Fig. 9A). mantADP binding to MD was modeled according to Reaction 7 (11).

![FIGURE 6. ATP binding to MD at low temperature.](image)

TABLE 1

| Kinetic parameters, step size, and actin sliding velocities in different myosin |
|-----------------------------------------------|
| **Chara (XI)** | **Rub (Fsk)** | **Dd (II)** | **Chick (Sm)** | **Chick (V)** | **Pig (VI)** | **Rat (I)** |
|----------------|---------------|-------------|---------------|--------------|-------------|------------|
| **Kinetic parameters in the presence of actin** |
| $k_{k +a}$ (μM s$^{-1}$) | 4.1 | 2–8 | 0.14 | 0.47 | 0.7 | 0.018 | 0.023 |
| $k_{k +a}$ (s$^{-1}$) | 2600 | 5000 | 450 | ND | 870 | >250 | 74 |
| $k_{ADP}$ (μM) | 260 | 120 | 94 | 5.0 | 0.9 | 8.8 | ND |
| $k_{-ADP}$ (μM) | >2,800 | ND | ND | 67 | 16 | ND | 6 |
| $k_{-ADP}$ (s$^{-1}$) (mantADP) | 2800 | ND | ND | 250 | ND | 12 | 5.6 |

**Kinetic parameters in the absence of actin**

| $k_{k +a}$ (μM s$^{-1}$) | 10 | 1.9 | 0.94 | 2.1 | 0.7 | ND | ND |
| $k_{k +a}$ (s$^{-1}$) | 36 | 3.2 | 0.7 | 2.9 | 0.7 | 0.27 | 0.1 |
| $k_{-ADP}$ (μM) | (530) | 130 | 24 | 50 | 750 | ND | ND |
| $k_{-ADP}$ (s$^{-1}$) | 1.9 | 1.6 | 1.5 | 1.1 | 4.6 | 1.0 | ND |
| $K_{ADP}$ (μM) | 2.7 | 1.4 | 0.9 | 1.9 | 1.2 | 6.4 | ND |

**Thermodynamic coupling between actin and ADP affinities ($K_{ADP}/K_{ADP}$)**

| Acceleration of ADP release by actin ($k_{-ADP}/k_{-ADP}$) | 1,000 | >360 | 280 | 35 | 12 | 0.88 | ND |

**Duration of the strongly bound state with actin (ms)**

| AM-ADP* | <0.36 | <2.0 | 4.0 | ~15 | 83 | 180 | 170 |
| AM* | 0.46 | 0.40 | 4.6 | ND | 1.2 | 3 | 27 |
| Total | <0.82 | <2.4 | 8.6 | 15 | 84.2 | 183 | 197 |

**Step size (nm)**

| 19 | 6 | 7.1 | 10 | 20 | 12 | 12 |

**Actin sliding velocity (μm/s)**

| 50 | 6 | 2.4 | 1.2 | 0.8 | 0.31 | 0.04 |
The mean value of the dissociation rate constant of mantADP from MD ($k_{-ADP}$) from five different MD preparations was 2.7 ± 0.4 s$^{-1}$. This value is similar to those of other myosins (Table 1) and ~1000-fold lower than that of acto-MD (Fig. 7). These results showed that ADP dissociation from MD is dramatically accelerated (~1000-fold) by actin binding.

ADP binding to MD was followed by the increase in fluorescence of mantADP (Reaction 8).

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The sliding velocity of actin filaments by MD was measured using an antibody-based version of the *in vitro* motility assay (31). MD was fixed to a glass surface by using an anti-c-**myc** monoclonal antibody. MD moved actin filaments at a velocity of 4.7 ± 0.3 μm/s at 25 °C and 6.3 ± 0.7 μm/s at 30 °C (Fig. 10). The velocity of MD used in this study was a little lower than that in the previous study (8.8 μm/s at 30 °C) (23). The difference in the velocity between the two MD constructs is probably caused by the difference in methods of attachment of MD to the glass surface. The sliding velocity by MD was also measured using mantATP. It was 2.3 ± 0.2 μm/s at 25 °C, which was about half of that using non-labeled ATP.

The actin sliding velocity by MD-Vneck was measured using an ordinary *in vitro* motility assay (56), except that the glass surface was not coated with nitrocellulose, because actin moved more smoothly on the non-coated glass than the coated surface. The velocity of MD-Vneck was 24 ± 1.2 μm/s at 30 °C (Fig. 10), which was four times faster than that of MD.

When the KCl concentration was raised to a physiological level (150 mM), the actin sliding velocities by MD and MD-Vneck increased to 8.7 μm/s ± 0.4 μm/s and 31 ± 1.5 μm/s (supplemental data, movie S1), respectively, at 30 °C (Fig. 10), showing that high ionic strength is suited for high velocity of *Chara* myosin.

**DISCUSSION**

*Duration of the Strongly Bound State—Chara corallina myosin* can move actin filaments ~10 times faster than the fast skeletal myosin. To unravel the molecular mechanism of how *Chara* myosin moves so fast, we investigated the kinetic properties of the MD of *Chara* myosin. According to the swinging lever arm theory, the actin sliding velocity is defined as the step size divided by the time spent in the strongly bound state ($t_s$) (5, 57). The step size of *Chara* myosin is 19 nm (58), which is three
times larger than that for fast skeletal muscle myosin (Table 1). However, this difference in step size is not enough to explain the difference in the sliding velocity. We examined, therefore, its kinetic parameters to see if $t_s$ is indeed small. Because attachment to actin and the following power stroke (a portion of the AM-ADP $P_i$ state) are very rapid, $t_s$ is mainly made up of the AM-ADP and the AM states (35, 59). Thus we determined the duration of these two states by measuring their time constants using stopped-flow apparatus.

The duration of the AM-ADP state is approximated as the inverse of the dissociation rate constant of ADP from acto-MD-ADP ($1/k_{ADP}$). $k_{ADP}$ was measured by monitoring the decrease in fluorescence of mantADP accompanying dissociation of mantADP from the acto-MD-mantADP complex. At physiological temperatures, the rate was too fast to be measured using our stopped-flow apparatus (dead time: 1.15 ms, detection limit: $\sim 1000 \text{ s}^{-1}$). Thus we measured $k_{ADP}$ at low temperatures, and the $k_{ADP}$ value at 25°C was estimated by extrapolating the $k_{ADP}$ values at low temperatures on the Arrhenius plot (Fig. 7). Similar to rat and rabbit cardiac myosins (59), a linear Arrhenius plot was observed. This plot yielded activation energies of 84 kJ/mol, which is also similar to those for rat and rabbit cardiac myosins (64 and 76 kJ/mol, respectively (59)). From the Arrhenius plot, the $k_{ADP}$ value at 25°C was estimated to be 2800 s$^{-1}$. Thus the duration of the AM-ADP state was 0.36 ms (1/2800 s, Table 1) when mantADP was used.

Although mantADP and ADP exhibit similar dissociation rates from acto-S1 for some myosin (14), the mantADP dissociation rate from acto-S1 is slower than ADP for other myosin (11). To test whether or not mantADP and ADP have similar kinetic parameter for *Chara* MD, we have done the in vitro motility assay using mantATP (see “Results”). The sliding velocity of actin filaments by MD using mantATP was 2.3 $\mu$m/s. According to the lever arm theory, actin sliding velocity is mainly determined by ADP dissociation rate from acto-MD and acto-MD dissociation rate caused by ATP binding to MD (5, 57). Because the association rate of mantATP for MD is higher than that of ATP (Fig. 5), the slower sliding velocity is likely due to the slow mantADP dissociation rate as compared with ADP. Thus $k_{ADP}$ value and the duration of the AM-ADP state were assumed to be $>2800 \text{ s}^{-1}$ and $<0.36 \text{ ms} (<1/2800 \text{ s}, Table 1), respectively, when using non-labeled ADP.

Owing probably to the large $k_{ADP}$ value, the equilibrium dissociation constant of acto-MD for ADP ($K_{ADP}$) was relatively large (Fig. 8 and Table 1), showing the low affinity of acto-MD for ADP.

The duration of the AM state is approximated as the inverse of the rate constant of ATP-induced acto-MD dissociation. The
rate constant of ATP-induced acto-MD dissociation is expressed as $K_1k_{-2}[ATP]/(1 + K_1[ATP])$ (Reaction 1) and is generally too fast to be measured for most fast myosins at a physiological ATP concentration ($\sim 3$ mM) and physiological temperature ($\sim 25$ °C). The rate constant of ATP-induced acto-MD dissociation can be approximated as $K_1k_{-2}$ at low ATP concentrations. The $K_1k_{-2}$ value at 25 °C was $4.1 \mu M^{-1} s^{-1}$ (Fig. 3). We also measured the rate constant of ATP-induced acto-MD dissociation at high ATP concentrations and low temperatures (Fig. 4A). Fitting the data to hyperbola at saturating ATP concentrations gave $k_{+2}$ values at low temperatures. The Arrhenius plot of the $k_{+2}$ values was linear (Fig. 4B) as seen in the case of fast skeletal myosin (35). This plot yielded an activation energy of 63 kJ/mol, which is similar to that for fast skeletal muscle myosin under similar conditions (53 kJ/mol (35)). The $k_{+2}$ value at 25 °C was estimated to be 2600 s$^{-1}$ by extrapolating this plot to 25 °C. From the $K_1k_{-2}$ value of 4.1 $\mu M^{-1} s^{-1}$ and the $K_{+2}$ value of 2600 s$^{-1}$, the $K_1$ value at 25 °C was calculated to be 1600 $M^{-1}$. Putting the physiological ATP concentration of 3 mM, the $k_{-2}$ value of 2600 s$^{-1}$, and the $K_1$ value of 1,600 $M^{-1}$ into $K_1k_{-2}[ATP]/(1 + K_1[ATP])$, the rate constant of ATP-induced acto-MD dissociation at physiological concentration of ATP (3 mM) and physiological temperature (25 °C) was calculated to be 2200 s$^{-1}$. Thus the duration of the AM state at physiological conditions was 0.46 ms ($\frac{1}{2200} s^{-1}$).

The calculated time spent in the strongly bound state ($t_s$) of MD is $<0.82$ ms ($<0.36$ ms of AM:ADP state + 0.46 ms of AM state). Because the rate constants of various myosins listed in Table 1 were obtained under a wide variety of conditions (temperature ranging from 20 to 25 °C and ionic strength ranging from 5 to 100 mM), we cannot rigorously compare the rate constants among various myosins. Nevertheless, the $t_s$ value of Chara myosin MD is extremely small, strongly suggesting that a short $t_s$, together with a large step size, are the main reasons for the fast movement of Chara myosin.

The ATPase cycle time, which is the inverse of the $V_{max}$ of the actin-activated ATPase activity of MD, was 1/ (390 s$^{-1}$) = 2.6 ms at 25 °C. Thus, the duty ratio (the proportion of $t_s$ to the ATPase cycle time) of MD is $<0.3$ ($<0.82$ ms/2.6 ms). Because the duty ratio of processive myosins must be more than 0.5 (normally, $>0.7$) (11), this result shows that Chara myosin is not a processive myosin, confirming our previous results (4).

The lever arm theory for Chara myosin movement was partially supported by the motility data of MD-Vneck: when the lever arm length increased ~8-fold that of MD (Fig. 1A), the velocity increased ~4-fold that of MD (Fig. 10) without increasing the ATP hydrolysis cycle rate (Fig. 2). This result shows that the long lever arm consisting mainly of six IQ motifs contributes to the high velocity, most likely due to its large step size. Although the velocity of MD-Vneck was strikingly high, it was still lower than that of native Chara myosin. This is most likely caused by the chimeric effect of the MD of Chara myosin, which is connected to the neck domain of myosins from different species (60).

Using optical tweezers, Kimura et al. (58) measured the dwell time of the unitary steps of native Chara myosin at 1 $\mu M$ to 1 mM ATP. From the distribution of dwell time, they estimated that the dwell time ($t_d$) for native Chara myosin was 100 ms even at 1 mM ATP, which could not explain the fast Chara myosin movement. However, the authors could not include data with a dwell time shorter than 20 ms in their analysis due to slow the responsiveness of their apparatus. If a reaction with a rate constant of 1000 s$^{-1}$ is observed using the apparatus with a dead time of 20 ms, most of the amplitude of the transient is lost and the remaining amplitude is almost 0% ($1/e^{1000 \times 0.02} = 2 \times 10^{-5}$) at the onset of the observation. In general, when using an apparatus with a dead time of a (s), only the reactions with rate constants of <1/a (s$^{-1}$) can be reliably approximated by a single exponential curve. In our case, because we used a stopped-flow apparatus with a dead time of 1.15 ms, only the reactions with rate constants of <500 s$^{-1}$ can be reliably measured. Thus the temperature of the apparatus was decreased to make rate constants <900 s$^{-1}$. Because the dead time of the apparatus used by Kimura et al. was 20 ms, only the reactions with rate constants of <50 s$^{-1}$ can be reliably measured. It is likely that such slow reactions (<50 s$^{-1}$) are not of intact myosins. Alternatively, Chara myosin light chains could drastically change the nature of Chara myosin MD. However, this seems unlikely because the ATPase cycle time of MD-Vneck (4.5 ms) was similar to that of MD (2.6 ms) and much shorter than the dwell time measured by Kimura et al. (100 ms).

**Striking Kinetic Features**—As shown in Table 1, actin sliding velocities for different type of myosins exhibit extraordinary divergence: velocity varies from 0.04 $\mu M/s$ (Rat myosin I) to 50 $\mu M/s$ (Chara myosin), a difference of more than 1000-fold. This large divergence in velocities is mainly caused by the large difference in the ADP dissociation rate constants from acto-myosin ($k_{-ADP}$): differences in $k_{-ADP}$ among the different myosins are also ~1000-fold, maximally (Table 1). On the other hand, there was no significant difference in ADP dissociation rate constants in the absence of actin ($k_{{ADP}}$) among the different types of myosins (Table 1). This means that actin markedly accelerates ADP release in fast myosins, whereas it does not do so in slow myosins. Actually, both the ratio $k_{-ADP}/k_{-ADP}$ (the acceleration of ADP release by actin) and the ratio $k_{{ADP}}/k_{+ADP}$ (termed the thermodynamic coupling between actin and ADP affinities) are large for fast myosins and small for slow myosins (Table 1). The ratio $k_{-ADP}/k_{-ADP}$ of Chara myosin is 1000 and the ratio $k_{{ADP}}/k_{+ADP}$ is 200 (Table 1). These values are the largest among myosins measured so far. These results suggest that actin binding quickly alters the structure of nucleotide binding site to accelerate ADP release.

Another noticeable feature of MD is an extremely fast ATP hydrolysis rate. The binding constant of MD for mantATP ($K_{+ADP}$) was $36 \mu M^{-1}$ s$^{-1}$ (Fig. 5B), which is >10 times larger than those of other myosins (Table 1). On the other hand, the binding constant of MD for mantADP ($k_{+ADP}$) is similar to those of other myosins (Fig. 9B and Table 1). These results exhibit a highly selective affinity of MD for ATP. When the binding rate was followed by the increase in tryptophan fluorescence, it was 9.8 $\mu M^{-1}$ s$^{-1}$ (Fig. 5A), which is also much higher than those of other myosins (Table 1). Possibly, a substantial portion of the high $K_{+ADP}$ value derives from the high value of the isomerization event ($k_{+2}$). The $K_{+ADP}$ obtained using tryptophan fluorescence was different from that...
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obtained using mantATP. Differences in $k_{-2}$ values between tryptophan fluorescence and mantATP were also observed in other myosins (37, 45). There are several possible reasons for this difference. One possibility is that the affinity of mantATP for MD is different from that of ATP for MD: the difference stems from the $K_i$ value. Another possibility is that the $k_{-2}$ value obtained using mantATP does not reflect isomerization, but only ATP binding. Anyway, although $K_i$ and $k_{-2}$ values are different between tryptophan and mantATP, both of the $K_i$ and $k_{-2}$ values of MD are much higher than those of other myosins (Table 1).

The rate of ATP hydrolysis ($k_{+3}$ and $k_{-3}$) could not be measured at physiological temperatures. Thus we measured this at 3 °C, and the rate was 530 s$^{-1}$ (Fig. 6). This result obviously shows the fast ATP hydrolysis rate of MD, because the $k_{+3}$ + $k_{-3}$ value of MD at 3 °C is similar to or much higher than those of other myosins at physiological temperatures (Table 1).

Taken together, Chara myosin has unique kinetic features suited for the fast movement, namely, a dramatic acceleration of other myosins at physiological temperatures (Table 1).

Acknowledgments—We thank Dr. Kazuo Satoh for helpful discussion and allowing us to use the stopped-flow apparatus at our convenience and Drs. Taro Q. P. Uyeda and Dietmar J. Manstein for critical reading of the manuscript and giving valuable comments.

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