The Kinetic Mechanism of Myo1e (Human Myosin-IC)*

Vol. 277, No. 24, Issue of June 14, pp. 21514–21521, 2002
Printed in U.S.A.

Mohammed El Mezgueldi §§, Nanyun Tang §§, Steven S. Rosenfeld ‡, and E. Michael Ostap §§
From the Departments of Physiology and The Pennsylvania Muscle Institute, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6085 and the Department of Neurology, University of Alabama, Birmingham, Alabama 35294

Myo1e is the widely expressed subclass-1 member of the myosin-I family. We performed a kinetic analysis of a truncated myo1e that consists of the motor and the single IQ motif with a bound calmodulin. We determined the rates and equilibrium constants for the key steps in the ATPase cycle. The maximum actin-activated ATPase rate ($V_{\text{max}}$) and the actin concentration at half-maximum of $V_{\text{max}}$ ($K_{\text{ATPase}}$) of myo1e are similar to those of the native protein. The $K_{\text{ATPase}}$ is low (~1 µM), however the affinity of myo1e for actin in the presence of ATP is very weak. A weak actin affinity and a rapid rate of phosphate release result in a pathway under in vitro assay conditions in which phosphate is released while myo1e is dissociated from actin. Actin activation of the ATPase activity and the low $K_{\text{ATPase}}$ are the result of actin activation of ADP release. We propose that myo1e is tuned to function in regions of high concentrations of cross-linked actin filaments. Additionally, we found that ADP release from actomyo1e is > 10-fold faster than other vertebrate myosin-I isoforms. We propose that subclass-1 myosin-I is are tuned for rapid sliding, whereas subclass-2 isoforms are tuned for tension maintenance or stress sensing.

Myosin-I motors are the single-headed, low molecular weight, membrane-associated members of the myosin superfamily (1). The molecular details of myosin-I function in the cell are not known, but myosin-I is thought to play important roles in cortical membrane tension (2), endocytosis and endocytic trafficking (3–6), signal transduction (7), and membrane ruffling (6, 8).

Myosin-I subunits are the most diverse of the unconventional myosins and are represented by at least two phylogenetically distinct subclasses based on sequence comparison of motor domains (9). Subclass-1 myosin-I isoforms have long tails that contain lipid binding (TH1), proline-rich (TH2), and Src homology-3 (TH3) domains. Subclass-1 isoforms were first discovered in lower eukaryotes (10) and are sometimes termed “amoeboid” isoforms. However, vertebrate members of this subclass have been identified (11, 12). Subclass-2 myosin-I isoforms have short tails that contain only TH1 domains and are also widely expressed (9).

The kinetic mechanisms of all characterized myosin-I isoforms follow the same pathway with the same biochemical intermediates (13–16). However, considerable kinetic variability exists within the myosin-I family. Key rate constants of isoforms from lower eukaryotes are significantly faster (~3–10-fold) than those of the vertebrate isoforms. The characterized lower eukaryote isoforms belong to subclass-1 (13), and the characterized vertebrate isoforms are members of subclass-2 (14–16), thus suggesting the possibility that kinetic properties correlate with the isoform subclass rather than with the specific organism. Measurement of the rate constants of a vertebrate subclass-1 isoform will help determine whether kinetic properties are conserved within the subclasses. Additionally, knowing the rate constants of the different isoforms allows us to understand the physical limitations and properties of myosin-I isoforms and thus provides insight into the cellular functions of the subclasses. Comparisons of the sequences of myosin-I subclasses may also help unravel the structural modifications required for kinetic tuning of all myosin motors.

Myo1e, also known as myr-3 (12) and human myosin-IC (11), is the subclass-1 myosin-I expressed in nearly all vertebrate cells. Localization studies show that myo1e is found in regions of high actin concentration (17, 18), and steady-state biochemical investigations suggest that myo1e is capable of cross-linking actin filaments via an ATP-insensitive actin binding site in its tail (19). This cross-linking results in triphasic actin activated ATPase activity. Unlike myosin-I from lower eukaryotes, the ATPase activity of myo1e appears to be regulated allosterically by its tail domain (19), and the ATPase activity is not predicted to be regulated by heavy chain phosphorylation by p21-activated kinase homologs (20). We measured all of the key rate constants in the myo1e ATPase pathway and found that, like subclass-1 isoforms from lower eukaryotes, the rate constants are significantly faster than the characterized vertebrate subclass-2 isoforms, thus suggesting conservation of kinetics and function within a single subclass across phylogenetic boundaries. However, we also found differences in the myo1e kinetics which may tune the motor for function in regions of high actin concentration.

EXPERIMENTAL PROCEDURES
Reagents, Proteins, and Buffers—N-Methylntraniloyl (mant)1 nu-
clide derivatives were purchased from Molecular Probes, and 2'-deoxymantATP (dmantATP) was synthesized as described by Hira-
sukka (21). ADP and ATP concentrations were determined spectro-
photometrically before each experiment by absorbance at 259 nm, $\epsilon_{259} = 15,400 M^{-1} cm^{-1}$. MantATP and mantADP concentrations were de-
termined by absorbance at 255 nm, $\epsilon_{255} = 23,300 M^{-1} cm^{-1}$ (21).

Rabbit skeletal muscle actin was prepared and gel filtered (22). Actin

1 The abbreviations used are: mant, N-methylntraniloyl; A, actin; CaM, calmodulin; dmant, 2'-deoxymant; DTT, dithiothreitol; M, myosin; P,BP, phosphate-binding protein; pyrene-actin, pyrenyl iodoacetamide-labeled actin.
concentrations were determined by absorbance at 290 nm, ε max = 26,600 M⁻¹ cm⁻¹ (23). Actin was labeled with pyrylen iodoacetamide (pyrene–actin) and gel filtered (24). All actin was stabilized with a molar equivalent of phallloidin (Sigma). Calmodulin (CaM) was expressed in bacteria and purified as described (25).

Steady-state and transient experiments were performed at 25 ± 0.1 °C in KMg50 buffer (10 mM imidazole, pH 7.0, 50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 1 mM DTT (26–28)) or KMg0 buffer (10 mM imidazole, pH 7.0, 1 mM MgCl₂, 1 mM EGTA, 1 mM DTT). Free CaM (1–3 μM) was included in all solutions.

Myo1e-I Expression and Purification—The cDNA for human myosin-IC (accession NM 004998), kindly provided by W. M. Bement (University of Wisconsin), was truncated at Glu720, generating a construct containing the motor domain and the only IQ motif (referred to as myo1e IQ throughout the paper). A FLAG peptide sequence was inserted at the C terminus and subcloned into the baculovirus transfer vector pVL1392 (Invitrogen). Recombinant baculovirus was generated using standard procedures.

Myo1e IQ with bound calmodulin was purified from Sf9 cells that were coinfected with virus containing recombinant myo1e IQ and CaM (Fig. 1). Four liters of log phase cells (2 × 10⁶ cells/ml) were infected and incubated at 27 °C for 60 h with shaking. Cells were harvested by centrifugation; suspended in lysis buffer (10 mM Tris, pH 7.0, 200 mM NaCl, 2 mM MgCl₂, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 0.01 mg/ml aprotinin, 0.01 mg/ml leupeptin), 2 mM MgATP, and 0.05% Igepal at 4 °C; and homogenized with five strokes in a Dounce homogenizer. Cell extract was centrifuged at 100,000 × g for 1 h. The supernatant was loaded onto anti-FLAG antibody columns (Sigma). Columns were washed with 5 column volumes of lysis buffer + 2 mM MgATP and 5 column volumes of lysis buffer. Myo1e IQ was eluted with 10 mM Tris, pH 8.0, 100 mM NaCl, 1 mM DTT, 5 μM CaM, 0.2 mg/ml FLAG peptide (Sigma), 0.01 mg/ml aprotinin, 0.01 mg/ml leupeptin. Eluted protein was loaded directly on to an 8-ml Mono Q column (Amersham Biosciences) equilibrated in column buffer (10 mM Tris, pH 8.0, 25 mM KCl, 1 mM DTT) and eluted with a linear 25 mM–1 M KCl gradient. The Mono Q column separated myo1e IQ from FLAG peptide, ADP, ATP, and free CaM. Fractions containing myo1e IQ were dialyzed versus storage buffer (10 mM Tris, pH 7.5, 100 mM KCl, 1 mM EGTA, 1 mM DTT, 50% glycerol), which concentrated the protein and allowed for storage at −20 °C. Quantitative densitometry showed that myo1e IQ was >95% pure, and the CaM:myo1e IQ ratio was 1:1. Approximately 2 mg of pure myo1e protein was obtained from 4 liters of cells.

Steady-state ATPase Activity and Sedimentation Assays—Steady-state ATPase activities were measured in KMg50 or KMg0 buffer at 25 °C using the NADH-coupled assay as described (27). Steady-state binding of 0.2 μM myo1e IQ to 0–50 μM actin was measured in KMg50 and KMg0 at 25 °C in the presence of 2 mM MgATP by ultracentrifugation assays (350,000 × g for 20 min). The fraction of actin-bound myosin was determined by assaysing the NH₂-EDTA ATPase activity of the supernatant (28).

Stopped Flow, Quenched Flow, and Kinetic Modeling—Transient kinetic measurements were made at 25 °C with an Applied Photophysics (Surrey, UK) SX.18MV stopped flow having a 1.2-ms dead time. Tryptophan fluorescence (λ ex = 295 nm) was measured using a 320 nm WG long pass emission filter (Oriel). A 400 nm long pass filter (Oriel) was used to monitor pyrene (λ ex = 365 nm), and mANTP and mANTP (λ ex = 295 nm) fluorescence. Usually three to five transients were averaged before nonlinear least square fitting. The time courses presented in the figures show the average of one to four individual traces. Transients were fitted to exponential functions using the software supplied with the stopped flow. Transient P release was measured using the coupled assay system containing the fluorescently labeled mutant of the phosphate-binding protein (PBP (30, 31)) with the stopped flow in sequential mixing mode using an excitation wavelength of 425 nm and a 440 nm long pass filter. The dead time of the instrument in this configuration was −2 ms. Quenched flow measurements were performed with a KinTek (State College, PA) RQF-3 apparatus. Errors reported are standard errors in the fits.

Kinetic modeling and simulations were performed using Scheme 1 of the actomyosin ATPase, where A is actin and M is myosin. Computer simulations were performed with KSIM (Neil C. Miller) or Berkeley Madonna (Robert I. Maciey and George F. Oster; University of California, Berkeley).

RESULTS

Steady-state ATPase and Actin Binding Parameters—The ATPase activities of subclass-1 myosin-I isoforms from all lower eukaryotes are activated by phosphorylation of serine or threonine at the TEDS site in an actin binding, surface loop by proteins that have homology to p21-activated kinases (20). Although myo1e IQ has serines immediately adjacent to the TEDS site, it is not predicted to be regulated by heavy chain phosphorylation (20). We were unable to phosphorylate myo1e IQ with Pak3 or Acanthamoeba myosin-I IQ heavy chain kinase (not shown), thus confirming the TEDS rule (20).

The steady-state ATPase activity of myo1e IQ is activated ~2-fold by actin filaments. Steady-state (Fig. 2) and single turnover measurements (not shown) gave rates of ~0.6 s⁻¹ in the absence of actin (Table I) in KMg50. The actin concentration at half-maximum of the steady-state ATPase rate (K ATPase) is 1.2 ± 0.36 μM, and the maximum ATPase rate (V max) at saturating actin is 1.2 ± 0.03 s⁻¹. The triphasic ATPase activity observed with native myo1e was not observed (19).

Steady-state actin binding measurements show that myo1e IQ is dissociated predominantly from 0–50 μM actin in KMg0 (Fig. 2) and KMg50 (not shown) in the presence of 2 mM MgATP. Therefore, in the presence of ATP, the predominant steady-state myo1e IQ intermediates have a very weak affinity for actin with
an equilibrium dissociation constant ($K_d$) greater than 50 μM.

**Myo1eIQ Binding to Actin Filaments**—An ~80% fluorescence quenching upon strong binding of myo1eIQ to pyrene-actin allowed us to monitor the association of myo1eIQ with actin in the absence (Reaction 1) and presence (Reaction 2) of MgADP. Data were modeled as

$$k_{-1} \quad \text{AM} = \quad A^* + M$$

**REACTION 1**

and

$$k_{-11} \quad \text{AM-ADP} = \quad A^* + M-ADP$$

**REACTION 2**

where $A^*$ represents the unquenched fluorescent state of pyrene-actin. Time courses of myo1eIQ and myo1eIQ-ADP binding to pyrene-actin follow single exponentials rates that depend linearly on the actin concentration (Fig. 2A). The apparent second order rate constant for myo1eIQ binding to actin obtained from the slope is $k_{-1} = 9.0 \pm 0.7 \mu M^{-1} s^{-1}$ (Table II). The apparent second order rate constant for myo1eIQ-ADP binding to actin is $k_{-11} = 1.2 \mu M^{-1} s^{-1}$ (Table II).

The rate of dissociation of myo1eIQ and myo1eIQ-ADP from pyrene-actin was measured by competition with 60-fold excess of unlabeled actin (Fig. 3B). Time courses fit single exponentials with rates $k_7 = 0.056 s^{-1}$ and $k_{11} = 0.065 s^{-1}$ (Table II). Dissociation equilibrium constants ($K_7$ and $K_{11}$) calculated from the dissociation and association rates ($k_{-7}/k_7$ and $k_{-11}/k_{11}$) were $K_7 = 6.2 nM$ and $K_{11} = 55 nM$ (Table II).

**MantATP Binding to Myo1eIQ and Actomyo1eIQ**—We used the enhancement in fluorescence of the ATP analog mantATP to measure the rate of nucleotide binding. Unlike skeletal muscle myosin-II (32), an intrinsic tryptophan fluorescence change upon MgATP binding was not detected. The transient increase
in mantATP fluorescence was best fit to two exponentials (data not shown). The two exponentials are not the result of the presence of multiple mantATP isomers (33) because a two exponential fluorescence increase was also detected when experiments were performed with dmantATP, which is a single-isomer preparation of mantATP. The amplitude of the fast exponential phase is 70–80% of the signal, and its rate is linearly related to the dmantATP concentration (Fig. 4A). We assumed a two-step ATP binding reaction,

$$K_1 \rightleftharpoons M + \text{ATP} \rightarrow \text{M(ATP)} \rightarrow \text{M-ATP}^*$$

where $K_1$ is a rapid equilibrium and $k_{-1}$ is a rate-limiting isomerization to the high fluorescence state of dmantATP (ATP$^*$). The apparent second order rate constant for dmantATP binding, given by the slope of the plot of the observed rate versus nucleotide concentration, is $K_1k_{-1} = 11 \pm 0.4 \mu M^{-1} s^{-1}$ (Fig. 4A). Fits of the slow exponential phase yielded an apparent second order rate constant $0.65 \mu M^{-1} s^{-1}$ (Fig. 4A, inset). It was proposed for skeletal muscle subfragment-1 that this slow rate reports the conformational change that accompanies ATP binding, given by the slope of the plot of the observed rate versus ATP concentration up to 70 $\mu M$ ATP and that linear fits of the data pass through the origin.

The ATP concentration dependence of $k_{\text{obs}}$ is shown for the full range of ATP concentrations. All stopped flow transients fit single exponentials. Pyrene fluorescence (solid line) and light scattering (dashed line) data are fit to rectangular hyperbolas. The inset shows pyrene fluorescence (top) and light scattering (bottom) transients obtained by mixing 0.25 $\mu M$ actomyoIQ with 100 $\mu M$ ATP fit to single exponential functions (smooth lines). B, expanded graph shows that $k_{\text{obs}}$ is linearly related to the ATP concentration up to 70 $\mu M$ ATP and that linear fits of the data pass through the origin.

A two-exponential fluorescence increase was also observed in the presence of actin. The amplitude of the fast exponential phase is 40–60% of the signal, and its rate is linearly related to the nucleotide concentration with an apparent second order rate constant 0.65 $\mu M^{-1} s^{-1}$ (Fig. 4B). The linear fit of the rates does not pass through the origin. The apparent second order rate constant determined from the slow exponential phase is 0.15 $\mu M^{-1} s^{-1}$ (Fig. 4 inset).

**ATP-induced Population of the Weakly Bound States—Pyrene-actin fluorescence was used to monitor the ATP-induced population of the weakly bound states (13, 34). Mixing ATP with pyrene-actomyoIQ resulted in an increase in fluorescence (Fig. 5A, upper trace in inset). Fluorescence transients were best fit to single exponentials at all ATP concentrations examined, and the rate of the fluorescence increase was hyperbolically related to the ATP concentration (Fig. 5A). The mechanism of ATP-induced fluorescence enhancement was modeled as shown in Reaction 4,

$$K_1' \rightleftharpoons A^* + \text{M-ATP}$$

where $K_1'$ is a rapid equilibrium, $k_{-1}'$ is a rate-limiting isomerization to the high fluorescence A*M-ATP state, and $k_{-1}'$ is the dissociation rate. The maximum rate is $k_{+1}' = 440 \pm 90 s^{-1}$ and $K_1' = 397 \pm 23 \mu M$ (Table II). The association rate constant for MgATP binding to actomyoIQ obtained from the initial slope ($K_1'k_{-1}'$) is $0.86 \pm 0.11 \mu M^{-1} s^{-1}$ (Fig. 5B) with a y intercept of zero.

Light scattering measurements were used to monitor the rate of actomyoIQ dissociation by MgATP (Fig. 5A). This measurement is different from the pyrene-actin measurements in that pyrene-actin fluorescence reports the population of the
attached and detached M·ATP states (Scheme 1). Mixing MgATP with actomyo1eIQ resulted in a rapid decrease in light scattering (Fig. 5A, lower trace in inset). The time courses follow single exponentials with rates that depend hyperbolically on the MgATP concentration (Fig. 5A). Fits to Reaction 4 yielded rate constants ($k_{21}$ = 334 ± 46 μM; $k_{22}$ = 439 ± 23 s$^{-1}$) nearly identical to those obtained with pyrene-actin (Table II). Therefore, the maximum rate of dissociation ($k_{5}$) upon ATP binding is limited by $k_{22}$ (Reaction 4).

ATP Hydrolysis—The rate of ATP hydrolysis in the absence of actin ($k_{3} + k_{-3}$; Scheme 1) was measured directly by quenched flow (Fig. 6). There is a rapid initial burst of ATP hydrolysis which fits a single exponential with a rate of 108 ± 14 s$^{-1}$ at 50 μM MgATP. The rate of ATP binding under experimental conditions is expected to be >500 s$^{-1}$ (Table II), so this measurement is not limited by the rate of nucleotide association. The presence of a phosphate burst indicates that the rate-limiting step occurs after ATP hydrolysis. The amplitude of the phosphate burst is 0.55 Pi/myosin (Fig. 6). If we assume that $B = K_{a}/(1 + K_{a})$ and $K_{a} = (k_{3}/k_{-3})$, then $K_{a} = 1.2, k_{-3} = 59$ s$^{-1}$, and $k_{3} = 49$ s$^{-1}$ (Table II).

Phosphate Release—Fluorescently labeled P$_{i}$BP was used to measure directly the rate of phosphate release ($k_{-4} + k_{4}$) in sequential mix, single turnover, stopped flow experiments (Fig. 7A (30)). Apyrase-treated myo1eIQ (2 μM) was mixed with 1 μM ATP, aged for 400 ms to allow for ATP binding, and mixed with 0–40 μM actin (Fig. 7B). P$_{i}$BP was included with the myo1eIQ and the actin to prevent transients resulting from phosphate released during the age time or phosphate contamination in the actin.

The time course of phosphate release in the absence of actin follows a single exponential with a rate of 1.5 s$^{-1}$ in KMg0 and KMg50 (Fig. 7A). There is no lag in the phosphate release transients (Fig. 7A, inset), indicating that phosphate release precedes ADP release. Unlike previously characterized myosins (28, 30), actin did not increase the rate of phosphate release (Fig 7B). This result is likely caused by the weak affinity of the M·ADP·P$_{i}$ state for actin (see “Discussion”).

ADP Association and Dissociation—The fluorescence increase of mantADP was used to determine the rate constants for ADP association to myo1eIQ and actomyo1eIQ (Fig. 8). In the absence of actin, time courses of fluorescence fit two exponential rates with approximately equal amplitudes. The fast phase was hyperbolically related to the nucleotide concentration (Fig. 8A) and modeled as a two-step binding reaction,

$$1/K_{a} \hspace{1cm} M + ADP \leftrightarrow M(ADP) \leftrightarrow M(ADP)^{2}$$

**REACTION 5**

where $^*$ indicates high mantADP fluorescence. The maximum rate is $k_{5} = 237 ± 13$ s$^{-1}$ and $K_{a} = 6.0 ± 0.9$ μM (Table II). The rate of the slow exponential phase shows only a slight nucleotide dependence and plateaus at $-11$ s$^{-1}$ at >10 μM mantADP (Fig. 8A, inset). The origin of this second exponential is not known, but it may report the population of a second M·ADP state (see “Discussion”).

In the presence of actin, time courses of fluorescence were best fit to single exponential rates that were linearly related to the nucleotide concentration (Fig. 9A). The signal to noise ratio of the fluorescence signal limited the range of testable mantADP concentrations, so we were not able to measure the maximum rate of mantADP binding ($k_{-5}$). Therefore, the apparent second order rate constant for mantADP binding was obtained by a linear fit to the data assuming the two-step binding model of Reaction 5 ($k_{-5}/K_{a} = 13 ± 1.8$ μM$^{-1}$ s$^{-1}$; Fig. 9A). The non-zero y intercept reveals a dissociation rate constant of $k_{-5} = 93 ± 5$ s$^{-1}$.

The rates of mantADP dissociation from myo1eIQ (Fig. 8B) and actomyo1eIQ (Fig. 9A, inset) were measured by competition with 250-fold excess of MgATP. Fluorescence time courses of mantADP dissociation fit two-exponential rates of 6.3 and 1.7 s$^{-1}$ in the absence of actin and a single exponential rate of $k_{-5} = 104 ± 4$ s$^{-1}$ in the presence of actin (Table II). The rate of MgADP dissociation from actomyo1eIQ is similar to that reported by the y intercept in the mantADP association experiment (Fig. 9A).

The affinity of MgADP for actomyo1eIQ was determined by competition experiments in which the ternary pyrene-
actomyo1eIQ-ADP complex was mixed with MgATP (Fig. 9B). Fluorescence transients fit single exponential rates that had a hyperbolic dependence on the MgADP concentration, suggesting that AM-D and AM states are in rapid equilibrium. Therefore, the dependence of $k_{obs}$ on MgADP concentration is as follows,

$$k_{obs} = \frac{k_0}{1 + (ADP/K_d)}$$  \hspace{1cm} (Eq. 1)

where $k_{obs}$ is the observed dissociation rate, $k_0$ is the dissociation rate in the absence of ADP ($K_d/k_2$ [ATP]; Table II), and $K_d$ is the dissociation equilibrium constant for ADP ($K_d$). Non-linear least square fits to the data yielded a dissociation equilibrium constant of $K_d = 3.9 \pm 0.29$ $\mu M$ (Table II), which is similar to the calculated value ($K_d = 8$ $\mu M$) using association and dissociation rate of mantADP.

DISCUSSION

Overview of the Myo1eIQ ATPase—Myo1eIQ has a high basal ATPase rate ($v_0$; Table I), yet all measured rate constant are >2-fold faster than $v_0$ (Table II), so we cannot assign a single step as rate-limiting. The slower turnover number is the result of two sequential steps (phosphate release and ADP release) with nearly equivalent rates. Kinetic modeling of the ATPase reaction using our determined rate constants (Table II) yields an ATPase rate ($v_0 = 0.65$ s$^{-1}$ in Kmg50), which is similar to the experimentally determined value ($v_0 = 0.58 \pm 0.04$ s$^{-1}$; Table I). The predominant steady-state intermediates in the absence of actin are M-ADP-P$_i$ and M-ADP ($P_i$).

**Fig. 9. Kinetics of mantADP binding to actomyo1eIQ.** A, rate of mantADP binding to 0.5 $\mu M$ actomyo1eIQ as a function of nucleotide concentration. The observed rates ($k_{obs}$) were obtained by fitting the stopped flow fluorescence transients at each nucleotide concentration to the sum of two exponentials. The solid line is the best fit of the fast $k_{obs}$ (○) to a rectangular hyperbola. The inset shows the nucleotide concentration dependence of the slow $k_{obs}$ (■). B, fluorescence transient showing the time course of mantADP dissociation from myo1eIQ. An equilibrated mixture of 10 $\mu M$ mantADP and 0.6 $\mu M$ myo1eIQ was mixed with 2.5 mM ATP. The smooth line shows the best fit of the data to a hyperbola described in Equation 1.

ATP Binding and Actomyo1eIQ Dissociation—The binding of ATP to myo1eIQ and actomyo1eIQ, as determined by dmantATP fluorescence, is fast. The rate of binding at physiological nucleotide concentrations is >100-fold faster than the rate-limiting step(s). Therefore, the nucleotide-free state of myo1eIQ is not significantly populated. The rate of population of the weak binding states as measured by pyrene-actin fluorescence and light scattering is also fast, and ATP-induced dissociation from
actin is limited only by the rate of ATP binding.

The rate of ATP binding to actomyo1eIQ as measured by mant fluorescence is ~4-fold faster than the rate measured by pyrene-actin fluorescence or light scattering. Additionally, the linear fit of the rates of dimantATP binding to actomyo1eIQ does not pass through the origin (Fig. 4B) but gives a y intercept of 25 s⁻¹. The value of the y intercept likely reports a reverse isomerization rate (kᵣ₋₂) of myo1eIQ. However, because the rate of ATP-induced dissociation of actomyo1eIQ is >400 s⁻¹ (Fig. 5) and essentially irreversible under the low actin concentration conditions of the experiment, a relatively slow reverse isomerization of the ATP binding step should not be observable. Therefore, the faster rate of mantATP binding and the observed reversal rate (kᵣ₋₂) are likely the result of an artifact because of the fluorescent modification of the 3' position of ATP. Although the dimantATP experiments probably do not report the true rate constants for ATP binding in the presence of actin, the experiments do reveal differences in the structural isomerization required for tight ATP binding in the presence and absence of actin. Additionally, because similar mant artifacts are not seen with other characterized myosins (e.g., 14), the experiments show that there are significant structural differences at the nucleotide binding sites of myosin-I isoforms.

Phosphate Release—The v₀ and V_max rates and the extent of actin activation of myo1eIQ (Table I) are similar to the values measured for native myo1 purified from rat liver (19), so the biochemical rate and equilibrium constants determined for S9-expressed myo1eIQ are likely identical to the native molecule. Therefore, the fast rate of Pᵢ release (k₋₄) is not an artifact of the expression system or of the expression of a truncated protein.

The rate of phosphate release from all previously characterized myosins is increased by actin binding (28, 30, 35). However, we did not detect actin activation of phosphate release from myo1eIQ. We propose that the lack of actin activation is caused by the low affinity of the M·ADP·Pᵢ state for actin. Other myosins bind actin in the presence of ATP with equilibrium dissociation constants <50 μM at low ionic strength conditions (36, 37), whereas myo1eIQ clearly binds with a dissociation constant >50 μM (Fig. 2). The ionic component of actin binding has been shown to be mediated by positive charges in surface loop-2 of myosin (36). Loop-2 of myo1e is shorter than those found in other characterized myosins (Fig. 10B) and does not contain the positively charged amino acids in the region that has been shown to be required for actin binding (Fig. 10B (36)). However, loop-2 of myo1e contains the KK region (Fig. 10B, underlined) shown to be crucial for actin activation of phosphate release (37). Therefore, it is possible that actin activates phosphate release from myo1eIQ, but we are not able to achieve high enough actin concentrations to populate the weakly bound AM·ADP·Pᵢ state. Weak actin binding may be a kinetic adaptation required for cellular function (see below).

It is possible that phosphate release by myo1eIQ is uncoupled from actin binding, so the very high basal rate of Pᵢ release (k₋₄) is also the rate when myo1eIQ is attached to actin (k₋₄). Why myo1e would evolve a mechanism that seemingly wastes ATP is not clear; however, other mechanisms for the suppression of the basal ATPase rate may exist. For example, the tail domain (which is not present in myo1eIQ) is thought to regulate the ATPase activity of myo1 allosterically (19). Further experiments are required to determine whether the myo1 tail regulates phosphate release directly.

**ADP Binding and Release**—Time courses of mantADP binding to myo1eIQ are best fit to two exponentials. The rate of the fast phase is hyperbolically related to the nucleotide concentration as expected for a two-step binding reaction (Reaction 6). The rate of the slow exponential does not show a significant nucleotide dependence and possibly reports the population of a second higher fluorescence state M·ADP·Pᵢ,

\[
\frac{1}{K_u} = M + ADP \xrightarrow{M(ADP)} M(ADP)^* \xrightarrow{k_{{-5a}}} M(ADP)^{**} \nonumber
\]

where * indicates the fluorescence state of myosin, k₋₅ₐ is the maximum rate of the fast phase, and k₋₅ₕ is the maximum rate of the slow fluorescence change. It has been shown previously that myosins exists in more than one ADP state (14, 38), so it is not surprising that we are able to detect multiple conformations of myo1eIQ. Dissociation of mantADP in the absence of actin also shows two exponentials that are likely related to these two states. However, a more detailed analysis of ADP binding is required to characterize these states better.

The similarity of the rates of Pᵢ release and ADP release in the absence of actin raises the possibility that the products are released simultaneously (Table II). However, if ADP and Pᵢ were released together, steady-state measurements of v₀ would be >2-fold faster (see above).

Time courses of mantADP binding to actomyo1eIQ fit single exponentials that are linearly related to the nucleotide concentration (Fig. 9A). A linear fit to the data does not pass through the origin but reveals a dissociation rate (k₋₅ₐ) of ~100 s⁻¹. This dissociation rate is nearly identical to the dissociation rate determined directly by displacing myo1eIQ-bound mantADP with unlabeled ATP (Fig. 9A, inset). The dissociation equilibrium constant (Kₑₑₐₑₑ) calculated by dividing the dissociation rate (k₋₅ₐ) by the association rate (k₋₅ₐ⁺) obtained from the mantADP experiments is within a factor of 2 of that determined from the pyrene-actin fluorescence experiments. Therefore, it is unlikely that the mantADP experiments are reporting artificially high rates as shown for dimantATP binding to actin (see above).

**Comparison with Other Myosin—I**—Myo1e is the first vertebrate subclass-1 myosin-1 to be characterized. Like the subclass-1 isoforms from Acanthamoeba (13), myo1e has relatively
large rate constants that are similar to those of skeletal muscle myosin-II (Table II). Most notably, the rates of ADP release from all subclass-1 isoforms are >10-fold faster than subclass-2 isoforms, i.e. the lifetimes of the AM-ADP states are >10 longer for subclass-2 isoforms (13–16). The rate of ADP release limits sliding velocity (39), so we propose that subclass-1 isoforms are better tuned for fast motility, whereas subclass-2 isoforms are better tuned for maintenance of force (14, 15).

A difference among the amoeba and vertebrate subclass-1 isoforms is that Acanthamoeba myosin-IA and -IB have low basal ATPase rates (<0.1 s⁻¹) and a large actin activation of the steady-state ATPase (> 50-fold (40)), whereas myo1e has an high basal ATPase rate and a low actin activation (Table I). Like myo1e, vertebrate subclass-2 isoforms also have high basal ATPase rates and relatively low actin activations (41). This might be an adaptation to the cellular environment of vertebrate cells, or it might be related to the regulation of myosin-I in these cells (8). A second difference between amoeba subclass-1 isoforms and myo1e is, like subclass-2 isoforms, myo1e has a very high ADP affinity (Kₐ = 10⁻μM). Therefore, it is possible that ADP release from both vertebrate subclasses is highly force-dependent (14, 15).

Relevance of Myo1e Kinetics to in Vivo Function—Like all other characterized myosin-I isoforms (13–16), myo1e is a low duty ratio motor, so under unloaded conditions it is predominately weakly bound or detached from actin filaments. Therefore, for myo1e to support motility, a high effective duty ratio must be created by bringing together locally high concentrations of myosin and actin. Such a mechanism is consistent with its observed cellular localization in regions of high F-actin concentration (17, 18).

The force-generating power stroke of myosin is thought to accompany phosphate release (42). Therefore, an ATPase pathway in which phosphate is released while myosin is detached from the actin filament (Fig. 10A, outlined pathway) would not generate force. It is possible that the power stroke occurs at a different step in the myo1e ATPase pathway (e.g. ADP release); however, this possibility is unlikely given the sequence and mechanistic conservation among myosin isoforms. We propose that the low actin affinity of myo1e in the presence of ATP is an adaptation to the high actin concentration environment in which myo1e functions (17, 18). Native myo1e cross-links actin filaments via an ATP-insensitive actin binding site in its tail, creating densely packed and cross-linked bundles (19). If the weak binding states (M-ATP and M-ADP-P) of myo1e had a high affinity, then ATP hydrolysis and the recovery stroke would occur while attached to actin (30). Therefore, an allosteric ATPase pathway would result in the reversal of myosin’s power stroke. To ensure that myo1e is detached from actin during the recovery stroke, a weak affinity must be maintained in the pre-force-generating states. We therefore predict that under the high actin concentrations of the cell, the M-ADP-P state will bind and release phosphate while attached to actin (Fig. 10A, gray pathway) as proposed for other myosins (43). Further experiments are required to confirm this prediction and to understand better the unique kinetic adaptations of all vertebrate myosin-I isoforms.

Acknowledgments—We thank Tianming Lin and Jade Poole for technical assistance; Li-Qiong Chen for constructing the expression virus; William M. Bement for providing the myo1e cDNA; and H. Lee Sweeney, Yale E. Goldman, and Enrique M. De La Cruz for helpful discussions and comments on the manuscript.

REFERENCES

1. Berg, J. S., Powell, B. C., and Cheney, R. E. (2001) Mol. Biol. Cell 12, 780–794
2. Dai, J., Ting-Beall, H. P., Hochmuth, R. M., Sheets, M. P., and Titus, M. A. (1999) Biophys. J. 77, 1168–1176
3. Gell, M. I., and Riezman, H. (1996) Science 272, 533–535
4. Jung, G., Wu, X., and Hammer, J. A., III (1996) J. Cell Biol. 133, 305–323
5. Rappos, G., Cordonnier, M. N., Tenza, D., Menichi, B., Durrbach, A., Louvard, D., and Coudrier, E. (1999) Mol. Biol. Cell. 10, 1477–1494
6. Noda, K., Peterson, M. D., Reddy, M. C., and Titus, M. A. (1995) J. Biol. Chem. 270, 1205–1212
7. Gillespie, P. G., Wagner, M. C., and Hudsath, A. J. (1993) Neuron 11, 581–594
8. Tang, N., and Ostap, E. M. (2001) Curr. Biol. 11, 1131–1135
9. Stoffler, D., and Bement, W. M. (2000) Int. Rev. Cytol. 200, 197–304
10. Pollard, T. D., and Korn, E. D. (1973) J. Biol. Chem. 248, 4682–4690
11. Bement, W. M., Wirth, J. A., and Moosiker, M. S. (1994) J. Mol. Biol. 243, 356–363
12. Stoffler, H. E., Ruppert, C., Reinhard, J., and Bahler, M. (1995) J. Cell Biol. 129, 819–830
13. Ostap, E. M., and Pollard, T. D. (1996) J. Cell Biol. 32, 1053–1060
14. Jones, J. D., Milligan, R. A., Pollard, T. D., and Ostap, E. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14332–14337
15. Coluccio, L. M., and Geeves, M. A. (1999) J. Biol. Chem. 274, 21575–21580
16. Geeves, M. A., Perreault-Micale, C., and Coluccio, L. M. (2000) J. Biol. Chem. 275, 21624–21630
17. Stoffler, H. E., Honnert, U., Baurer, C. A., Hofer, D., Schwarz, H., Muller, R. T., Drenckhahn, D., and Bahler, M. (1998) J. Cell Sci. 111, 2779–2785
18. Skowron, J. F., Bement, W. M., and Moosiker, M. S. (1998) Cell Motil. Cytoskeleton 41, 308–324
19. Stoffler, H. E., and Bahler, M. (1998) J. Biol. Chem. 273, 14605–14611
20. Bement, W. M., and Moosiker, M. S. (1995) Cell Motil. Cytoskeleton 31, 87–92
21. Hiratsuka, T. (1983) Biochim. Biophys. Acta 742, 496–508
22. Spudich, J. A., and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871
23. Hoek, T. W., and Tomkiewicz, K. (1976) Acta Biochim. Pol. 23, 60–74
24. Pollard, T. D. (1984) J. Cell Biol. 99, 769–777
25. Putkey, J. A., Slaughter, G. B., and Means, A. R. (1985) J. Biol. Chem. 260, 4764–4772
26. De La Cruz, E. M., Wells, A. L., Sweeney, H. L., and Ostap, E. M. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 12992–12996
27. De La Cruz, E. M., Wells, A. L., Sweeney, H. L., and Ostap, E. M. (2000) Biochemistry 39, 14196–14202
28. Chalovich, J. M., and Eisenberg, E. (1982) J. Biol. Chem. 257, 2432–2437
29. White, H. D., Belknap, B., and Webb, M. R. (1997) Biochemistry 36, 11828–11836
30. Xing, J., Wrighers, W., Jefferson, G. M., Stein, R., Cheung, H. C., and Rosenfeld, S. S. (2000) J. Biol. Chem. 275, 35413–35423
31. Johnson, K. A., and Taylor, E. W. (1978) Biochemistry 17, 3432–3442
32. Woodward, S. K. A., Eccleston, J. F., and Geeves, M. A. (1991) J. Biol. Chem. 266, 32373–32381
33. Furch, M., Geeves, M. A., and Manstein, D. J. (1998) Biochemistry 37, 6317–6326
34. Furch, M., Geeves, M. A., and Manstein, D. J. (1998) Biochemistry 37, 6317–6326
35. Furch, M., Geeves, M. A., and Manstein, D. J. (1998) Biochemistry 37, 6317–6326
36. Furch, M., Geeves, M. A., and Manstein, D. J. (1998) Biochemistry 37, 6317–6326
37. Furch, M., Geeves, M. A., and Manstein, D. J. (1998) Biochemistry 37, 6317–6326
The Kinetic Mechanism of Myo1e (Human Myosin-IC)
Mohammed El Mezgueldi, Nanyun Tang, Steven S. Rosenfeld and E. Michael Ostap

J. Biol. Chem. 2002, 277:21514-21521.
doi: 10.1074/jbc.M200713200 originally published online April 8, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M200713200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 43 references, 25 of which can be accessed free at
http://www.jbc.org/content/277/24/21514.full.html#ref-list-1