Myosin X Is a High Duty Ratio Motor*

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Myosin X is expressed in a variety of cell types and plays a role in cargo movement and filopodia extension, but its mechanoenzymatic characteristics are not fully understood. Here we analyzed the kinetic mechanism of the ATP hydrolysis cycle of acto-myosin X using a single-headed construct (M10IQ1). Myosin X was unique for the weak “strong actin binding state” (AMD) with a Kd of 1.6 μM attributed to the large dissociation rate constant (2.1 s⁻¹). Vmax and KATPase of the actin-activated ATPase activity of M10IQ1 were 13.5 s⁻¹ and 17.4 μM, respectively. The ATP hydrolysis rate (>V100 s⁻¹) and the phosphate release rate from acto-myosin X (>100 s⁻¹) were much faster than the entire ATPase cycle rate and, thus, not rate-limiting. The ADP off-rate from acto-myosin X was 23 s⁻¹, which was twice as large as the Vmax. The P1-burst size was low (0.46 mol/mol), indicating that the equilibrium is significantly shifted toward the prehydrolysis intermediate. The steady-state ATPase rate can be explained by a combination of the unfavorable equilibrium constant of the hydrolysis step and the relatively slow ADP off-rate. The duty ratio calculated from our kinetic model, 0.6, was consistent with the duty ratio, 0.7, obtained from comparison of Km,ATPase and Km,motility. Our results suggest that myosin X is a high duty ratio motor.

Myosin is an actin-based mechanoenzymatic protein that plays a critical role in various cell motile processes. It is now clear that myosin constitutes a diverse superfamly in which at least 11 subclasses are present in mammals (1–4). Among them, myosin X is expressed ubiquitously in various mammalian tissues (5, 6). Based upon the deduced amino acid sequence, it is predicted that myosin X consists of a conserved motor domain, three IQ motifs that function as light chain sequence, it is predicted that myosin X consists of a conserved motor domain, three IQ motifs that function as light chain binding sites, a coiled-coil domain, and a unique tail domain (6). The tail domain composed of three PH1 domains, one MyTH4 (myosin tail homology 4) domain, and one FERM domain is thought to function as the myosin X binding partner protein anchoring site. It has been reported that myosin X is present at the edge of lamellipodia, membrane ruffles, and the tip of filopodia in cultured cells, suggesting that myosin X plays a role in regions where actin is in dynamic reorganization (6). Myosin X associates with the cytoplasmic domain of β1-integrin at the FERM domain and translocates β1-integrin to the tip of filopodia, thus playing a role in adhesion of filopodia that helps to extend filopodia (7). Myosin X also binds to Mena/VASP and moves them from the root to the tip of filopodia (8). Because Ena/VASP antagonizes the ability of capping proteins to inhibit actin polymerization at the barbed ends in vitro (9), it is thought that the distribution of Ena/VASP at specific cellular locations may regulate the elongation of actin filaments that is correlated with the change in cytoskeletal structure and cell shape. Taken together, these findings have suggested myosin X is a cargo transporter.

Whereas the motor domain of myosin among the diverse superfamly members is highly conserved, it has been realized that the characteristics of the motor function varies uniquely from one to another. One of the most important issues is the processivity. Myosin Va was first identified as a processive myosin that can move on actin filaments for a long distance without dissociation (10–13). Clearly, the processive nature is suitable for the cargo transporting motors, and there is evidence that myosin Va transports melanosomes in melanocyte (14, 15). On the other hand, conventional myosin is a typical non-processive myosin that spends the majority of time during the ATPase cycle dissociated from actin. An advantage of non-processive myosin is that many molecules can simultaneously interact with one actin filament thus producing a large force without interfering with each other. Actually, a number of myosin II molecules in a thick filament interact with an actin filament and produce a large force. For myosin X, a previous biochemical study has suggested that myosin X is not a processive motor although the duty ratio of myosin X, the fraction of time that myosin spends in a strong actin binding form, may be higher than conventional myosin based on the surface density dependence of actin gliding velocity (16).

The aim of this study was to clarify the mechanoenzymatic characteristics of myosin X by analyzing the acto-myosin X ATPase reaction. The critical kinetic steps of the actomyosin X ATP hydrolysis mechanism were studied by various techniques, and the duty ratio was estimated. The results suggest that myosin X is a high duty ratio motor.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and modifying enzymes were purchased from New England Biolabs (Beverly, MA). Purine nucleoside phosphorylase (PNPase), 7-methylguanosine (MEG), phosphonopyruvate, and pyruvate kinase were obtained from Sigma. Actin was prepared from rabbit skeletal muscle according to Spudich and Watt (17) and dissolved in a buffer containing 25 mM KCl, 25 mM Hepes-KOH, pH 7.5, 5 mM MgCl₂, 1 mM EDTA. Pyrene-actin was prepared as described (18). 7-Diethylamino-3-((((2-maleimidyl)ethyl)amino)carbonyl)coumarin-labeled phosphate-binding protein (MDCC-PBP) was prepared as described (19, 20). Recombinant calmodulin was expressed in Escherichia coli and purified as described previously (21).

Generation of the Expression Vectors for Myosin X Constructs—Bovine myosin X cDNA encoding Met¹ –Gln⁷⁷³ was subcloned into modified
pFastBac1 baculovirus transfer vector containing a hexahistidine tag sequence at the 3’-end of the polylinker region as described previously (M10CC) (16). KpnI sites were created at nucleotides 2319 and 3183 of M10IQ1. The plasmid cDNA was digested with KpnI to excise the nucleotides 2320–3162 and ligated to produce an M10IQ1 construct encoding the entire motor domain plus one IQ domain of myosin X.

Expression and Purification of Myosin X Protein—To express the recombinant M10IQ1 protein, Sf9 cells (about 4–5 × 10^9 cells) were co-infected with two viruses expressing the M10IQ1 heavy chain and calmodulin. The infected cells were cultured for 3 days at 28 °C. Cells were harvested and washed with 200 ml of NaCl, 50 mM Tris-HCl, pH 7.5, and 5 mM EGTA. Cells were then lysed by sonication in 20 ml of lysis buffer (30 mM Hepes-KOH, pH 7.5, 25 mM KCl, 0.2 mM EGTA, 2 mM MgCl₂, 1 mM ATP, 1 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml trypsin inhibitor). After centrifugation at 300,000 × g for 10 min, the supernatant was incubated with 300 μl of nickel-nitriotropic acid agaroose (Ni-NTA) (Qiagen, Hilden, Germany) in a 14-ml conical tube on a rotating wheel for 10 min to immobilize M10IQ1. The precipitate was then washed on a column (1 × 10 cm) and washed with 10 ml of buffer A (20 mM Hepes-KOH/10 mM imidazole-HCl, pH 7.5, 50 mM KCl, 0.1 mM EGTA, 5 μg/ml leupeptin, and 1 mM 2-mercaptoethanol). M10IQ1 was eluted with buffer B containing 0.2 M imidazole-HCl, pH 7.5. The M10IQ1 was dialyzed against buffer B (25 mM KCl, 25 mM Hepes-KOH, pH 7.5, 5 mM MgCl₂, 1 mM EGTA). The purified M10IQ1 was stored on ice and used within 2 days.

Typically, 1.5 mg of M10IQ1/CaM was obtained. Protein concentration was determined by the Bradford method by employing bovine serum albumin as control, and corrected by purity of the sample determined by densitometry analysis, assuming degradation of the protein is negligible. [2,8-3H]ADP/VO₄ was employed to determine the concentration of active M10IQ1. The active motor concentration was typically 70–80% of that estimated by Bradford method followed by densitometry primarily because of overestimation of the M10IQ1 concentration using bovine serum albumin as a control with possible minor contamination of dead M10IQ1 during the purification.

Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was carried out on a 7.5–20% polyacrylamide slab gel using the discontinuous buffer system of Laemmli (22). After staining the gel with Coomassie Brilliant Blue, the purity of the M10IQ1 preparation was determined by densitometry analysis of the SDS-PAGE gel.

ATPase Assay—The steady-state ATPase activity was measured at 25 °C in the presence of 2 mM ATP with an ATP regeneration system (40 units/ml pyruvate kinase and 2 mM phosphoenolpyruvate). The reaction was carried out in buffer B plus 0.2 mg/ml calmodulin at 25 °C. The liberated pyruvate was determined as described (23). The error is the standard error in three independent preparations.

In Vitro Motility Assay—The actin gliding velocity was measured by in vitro motility assay. A coverslip coated with nitrocellulose and coated with bovine serum albumin and penta-His monoclonal antibody (Qiagen). M10IQ1 was then applied to the coverslip. The movement of the rhodamine-labeled actin filaments was observed in 25 °C buffer C (25 mM KCl, 25 mM imidazole-HCl, pH 7.5, 5 mM MgCl₂, 1 mM EGTA, 143 mM 2-mercaptoethanol, 36 μg/ml catalase, 4.5 mg/ml glucose, 216 μg/ml glucose oxidase, 40 units/ml pyruvate kinase, 2 mM phosphoenolpyruvate) with various concentration of ATP. De-labeled F-actin was prepared as previously described (24). The error bar shows the standard deviation of ~100 data points obtained from movement of 5–10 different actin filaments.

Actin Co-sedimentation Assay—The binding of M10IQ1 with actin in the presence of ADP was determined by employing 1 μM M10IQ1 and 5 μM F-actin in buffer B plus 0.2 mg/ml calmodulin and 0.1 mM ADP, and centrifuged at 300,000 × g at 25 °C for 10 min. The pellet was dissolved with SDS-PAGE buffer, and the supernatant and the pellet were analyzed by SDS-PAGE with Coomassie Brilliant Blue staining.

Quench Flow Measurement—Quench Flow measurement was performed in buffer B plus 0.2 mg/ml calmodulin at 25 °C using a KinTek RQF-3 apparatus (KinTek Co., Clarence, PA) using [γ-32P]ATP as described (25). The error is the standard error from three independent experiments.

Stopped-flow Measurements—Kinetic measurements were performed in buffer B plus 0.2 mg/ml calmodulin at 25 °C using a KinTek SF-2001 apparatus with a 75-watt xenon lamp. Mant nucleotides were excited at 360 nm, and the fluorescence was monitored using a 420-nm cut-off filter. Pyrene-actin was excited at 365 nm with a 365-nm band-pass filter, and the fluorescence was monitored using a 400-nm cut-off filter. Intrinsc tryptophan residues in M10IQ1 were excited at 295 nm, and the fluorescence was monitored using a 340-nm cut-off filter.

RESULTS

Expression and Purification of the Myosin X Construct—We produced a bovine myosin X construct having the motor domain and the first IQ domain (Met¹–Gln⁷⁷³). This construct (M10IQ1) was used in the present study to avoid the possible complexity arising from the interhead interaction. The M10IQ1 was expressed using the baculovirus expression system to together with calmodulin in Sf9 cells and purified with Ni²⁺-ATPase activity of M10IQ1. ATPase activity was measured as described under "Experimental Procedures." The solid curve is the best fit to Michaelis-Menten kinetics with V_max and K_MATP of 13.5 head⁻¹ s⁻¹ and 17.4 μM, respectively. The broken line is simulated based on rate constants obtained in the present study (see "Discussion"), which gave V_max and K_ATP of 13.3 s⁻¹ and 17.7 μM, respectively. The error bars represent the standard error from three independent experiments. C, in vitro motility activity of M10IQ1. In vitro motility assay was performed as described under "Experimental Procedures" with 0.1 mM ATP. The velocity was 0.17 ± 0.03 μm s⁻¹.

MDCC-PBP was excited at 436 nm with a 436-nm band-pass filter, and the fluorescence was monitored using a 450-nm cut-off filter. 3 μM MDCC-PBP, 0.01 units/ml PNPase, and 0.3 mM MEG were pre-included in all solutions. Light scattering at 550 nm was observed to monitor dissociation of acto-M10IQ1. The volume ratio of the syringe was 1:1 in all single mixing experiments and 1:1.1 in double mixing experiments. Curve fitting was done using the KinTek SF-2001 software. The error represents the standard error from 3–10 independent experiments.

Kinetic Simulation—Kinetic simulations were performed using STELLA v8.1.1 software (iseesystems, Lebanon, NH).

Expression and Purification of the Myosin X Construct—We produced a bovine myosin X construct having the motor domain and the first IQ domain (Met¹–Gln⁷⁷³). This construct (M10IQ1) was used in the present study to avoid the possible complexity arising from the interhead interaction. The M10IQ1 was expressed using the baculovirus expression system to together with calmodulin in Sf9 cells and purified with Ni²⁺-agarose affinity column. As expected from the design of the construct, the isolated protein had an apparent molecular mass of 90 kDa co-purified with a low molecular mass peptide (Fig. 1A). The mobility of the low molecular mass peptide on an SDS-PAGE increased with Ca²⁺, suggesting that it is calmodulin (not shown). The amount of bound calmodulin to M10IQ1 seemed to be low. We think that the bound calmodulin was partially lost during the purification steps. We observed that the addition of calmodulin to the purified M10IQ1 increased the actin-activated ATPase activity, suggesting that the IQ domain of the purified M10IQ1 is not completely occupied with calmodulin. Based upon the increase in the ATPase activity,
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the apparent dissociation constants of calmodulin for M10IQ1 were estimated to be 2.7 ± 1.2 μM and 1.4 ± 0.2 μM in the presence (0.1 mM) and the absence of Ca^{2+}, respectively. Hence, we added 0.2 mg/ml calmodulin to the M10IQ1 sample in all experiments in this study. We also tried co-expression of M10IQ1 with calmodulin-like protein (CLP), since CLP has been reported as a light chain of myosin X, which preferentially binds to the third IQ domain in the presence of Ca^{2+} (26).

Despite the high expression levels in the Sf9 cell, which were comparable to that of calmodulin, the co-expressed CLP was not co-purified with M10IQ1 (not shown). The result is consistent with the previous finding, when we consider the lower affinity of CLP to the first IQ motif and the EGTA condition for the purification procedure. Therefore, we examined M10IQ1 having only calmodulin as the light chain in this study.

Steady-state ATPase Activity and in Vitro Actin Translocating Activity of M10IQ1—Fig. 1B shows the actin-activated ATPase activity of M10IQ1 as a function of the actin concentration. The ATPase activities at various actin concentrations were fitted well with a Michaelis-Menten equation to yield V_{max} and K_{ATPase} of 13.5 ± 1.6 s^{-1} and 17.4 ± 5.2 μM, respectively. We also performed an in vitro motility assay on the M10IQ1 construct to see whether the M10IQ1 retains the actin translocating activity. The velocity was 0.17 ± 0.03 μm s^{-1} (Fig. 1C) with plus-end movement directionality (not shown). The results suggest that the truncation does not impair the motor function of myosin X. We also found that the addition of exogenous calmodulin (>0.05 mg/ml) greatly improved the movement probability of actin filaments in the in vitro motility assay, suggesting that the binding of light chain to the IQ domain is essential for the motor function of myosin X, and the affinity of calmodulin to the first IQ domain is relatively low. This is consistent with the results from the calmodulin-dependent ATPase assay.

MgATP Binding to M10IQ1 and Acto-M10IQ1—Fig. 2 shows the rate of Mant-ATP binding to M10IQ1 as a function of Mant-ATP concentration. The increase in the fluorescence intensity of Mant-ATP followed single exponential kinetics for both M10IQ1 and acto-M10IQ1 (Fig. 2, inset). The observed rate constants (k_{obs}) were obtained by fitting the time course of the change in fluorescence of Mant-ATP upon the binding to M10IQ1 at each concentration to a single exponential. The insets show typical recordings of Mant-ATP (1 μM) binding to M10IQ1 (0.25 μM) in the absence (A) or the presence of 1 μM actin (B). The concentrations indicate the final concentrations after mixing. Apparent second order binding rate constants of 4.7 μM^{-1} s^{-1} and 3.4 μM^{-1} s^{-1} were obtained for Mant-ATP binding to M10IQ1 and acto-M10IQ1, respectively. The solution condition was 25 mM KCl, 25 mM Hepes-KOH, pH 7.5, 5 mM MgCl$_2$, 1 mM EGTA, and 0.2 mg/ml calmodulin at 25 °C. The error bars represent the standard error from 3–6 independent experiments.

ATP-induced dissociation of acto-M10IQ1 and the formation of weak actin binding form of M10IQ1. ATP-induced acto-M10IQ1 dissociation and weak actin binding formation were monitored by light scattering (square) and pyrene-actin fluorescence (circle). The inset shows the typical recordings of the time course of light scattering change (lower trace) and pyrene fluorescence change at 25 μM ATP (final). Apparent rates were obtained by single exponential fitting. At high ATP conditions, the initial signal changes were missed because of the dead time of the instrument. However, ∼30–40% of the total signal could be monitored after the dead time even at the rate constant of 700 s^{-1}, and this was sufficient to determine the rate constant. Concentrations of M10IQ1 and actin (or pyrene-actin) after mixing were both 0.25 μM. Conditions were the same as described in the legend to Fig. 2. The error bars represent the standard error from 3–6 independent experiments.

MgATP-induced Population of Weakly Bound Acto-M10IQ1—Pyrene-labeled actin was employed to monitor the ATP-induced population of weakly bound acto-M10IQ1 state. Rapid mixing of MgATP to pyrene-acto-M10IQ1 resulted in an increment of pyrene fluorescence as is observed for other unconventional myosins (27–29). The time course of fluorescence transient observed was best fitted to a single exponential (Fig. 3, inset), and the apparent rate constants at various MgATP concentrations showed a hyperbolic relationship against MgATP concentration (Fig. 3). The binding rate constant of MgATP to acto-M10IQ1 (k_{b+}) can be obtained from the initial slope. The value (2.7 ± 0.4 μM^{-1} s^{-1}) was consistent with that obtained using Mant-ATP (Fig. 2). The maximum rate constant estimated from the hyperbolic curve fitting (k_{-b}) was 782 ± 74 s^{-1}, which was more than 50-fold larger than the V_{max} of the steady-state ATPase activity of acto-M10IQ1. Therefore, the ATP binding step and the following isomerization step (k_{-f}) are not rate-limiting for the acto-M10IQ1 ATPase cycle as for other myosins characterized so far.

Dissociation of Acto-M10IQ1 by MgATP—The dissociation of acto-M10IQ1 was monitored by measuring the decrease in light scattering intensity upon mixing acto-M10IQ1 with MgATP (Fig. 3). The time course of the signal change was fitted with single exponential kinetics (Fig. 3, inset), and the apparent rate constants of acto-M10IQ1 dissociation were hyperbolically increased with ATP concentration to yield the maximum value of 885 ± 36 s^{-1}. The hyperbola was almost identical to that obtained from the change in pyrene fluorescence intensity, suggesting that the dissociation of acto-M10IQ1 (k_{-f}, k_{-p} pathway) is a rapid process.
and yielded a maximum rate constant of 395 \( s^{-1} \). \( k_1 \) increased with ATP to show a hyperbolic dependence (Fig. 4) upon addition of ATP, and the tryptophan fluorescence was monitored. Apparent rates were obtained by single exponential fitting and plotted against ATP concentration. The inset shows a typical recording of the experiment at 2.5 \( \mu M \) (final) ATP. The maximal rate of 395 \( s^{-1} \) was obtained. Conditions were the same as described in the legend to Fig. 2. The error bars represent the standard error from 3–5 independent experiments.

Enhancement of Intrinsic Tryptophan Fluorescence Intensity—Myosin X contains a conserved tryptophan residue (Trp481) located at the rigid relay loop. This residue is equivalent to Trp210 of skeletal muscle myosin II that has been shown to be the largest contributor to the enhancement of intrinsic tryptophan associated with ATP binding and hydrolysis. The intrinsic tryptophan fluorescence intensity of M10IQ1 increased with ATP and the increase followed single exponential kinetics (Fig. 4, inset). The apparent rate constants increased with ATP to show a hyperbolic dependence (Fig. 4) and yielded a maximum rate constant of 395 \( s^{-1} \). The MgATP binding rate constant obtained from the initial tangent of the hyperbola was 4.4 \( s^{-1} \), which was consistent with the value obtained from the Mant-ATP binding experiment (Fig. 2). We assume that the intrinsic tryptophan enhancement of M10IQ1 by MgATP monitors predominantly the hydrolysis step \( (k_{-3} + k_{-1}) \), because the observed maximal rate constant of 300–400 \( s^{-1} \) was less than half of that of the maximal ATP binding rate \( (k_{1} - 800 s^{-1}) \) determined by the ATP-induced pyrene fluorescence change. Because the ATP binding rate constant in the absence of actin is even greater than that in the presence of actin (suggesting \( k_{-2} > k_{-1} \) if \( k_{1} = -k_{-1} \)), the intrinsic tryptophan enhancement predominantly monitors the ATP binding but not the following hydrolysis step, the maximal rate should be \( k_{-2} \), which should be similar to or larger than \( k_{-2} \) \( (800 s^{-1}) \).

Quench Flow Measurements of ATP Hydrolysis by M10IQ1—It is known that myosin forms a myosin/ADP/P ternary complex (MDP) after ATP hydrolysis. Because the protein-bound phosphate is released from myosin by acid quenching of the ATPase reaction, rapid initial phosphate release is observed when the reaction is stopped by acid (P burst). Fig. 5 shows the time course of the P\(_1\) release of M10IQ1 ATPase reaction measured by a quench flow experiment. A single turnover experiment was carried out to determine the equilibrium of the hydrolysis step (Fig. 5A). 1 \( \mu M \) \( [\gamma^{32P}] \text{ATP} \) was mixed with 2 \( \mu M \) M10IQ1 so that all given ATP was bound to the ATP binding site of myosin X. \( P_1 \) was rapidly released from acto-M10IQ1 followed by a slow \( P_1 \) release. The fast phase represents the initial rapid ATP hydrolysis by M10IQ1. The slow phase (0.06 \( s^{-1} \)) corresponds to the apparent \( P_1 \) release rate \( (k_{1-4} \text{ obs}) \). The presence of the slow \( P_1 \) release phase suggests that a certain population of M10IQ1 is present as a prehydrolysis step (MT). From the ratio of the fraction of the fast phase to the slow phase, the \( P_1 \) burst size of 0.46 \( \pm 0.02 \) was observed. This result indicates that the equilibrium of the MT-MDP step is significantly shifted to the prehydrolyzed form. The rate of ATP hydrolysis was also measured with higher ATP concentrations (Fig. 5B). The initial \( P_1 \) burst rates were 65 \( s^{-1} \) and 124 \( s^{-1} \) in the presence of 10.7 \( \mu M \) ATP and 20.9 \( \mu M \) ATP, respectively. The observed rate constants were limited by ATP binding rate at a given ATP concentration, suggesting that the ATP hydrolysis rate \( (k_{1-3} + k_{-2}) \) is greater than 120 \( s^{-1} \).

Phosphate Release Rate—Fluorescently labeled phosphate-binding protein (MDCC-PBP) was used to monitor the rate of phosphate release in the presence or absence of actin (Fig. 6). The fluorescence increased upon binding of released phosphate, and it followed single exponential kinetics in the absence of actin. The observed rate, 0.06 \( \pm 0.02 s^{-1} \) \( (k_{1-4} \text{ obs}) \), was consistent with the rate obtained from the quench flow experiment (Fig. 5A). In the presence of actin, however, the fluorescence increment followed double exponential kinetics as reported recently (30). The fast phase was actin concentration-dependent as expected from the kinetics scheme (Scheme 1). We could not see any sign of curvature within the experimentally achievable actin range we tested. The slope gives us a second order rate constant for MDP-AMDP transition \( (k_{-5} [K_p \text{ obs}]) \) and it was 0.71 \( s^{-1} \) \( (k_{1-4} \text{ obs}) \). The maximum \( P_1 \) release rate in the presence of actin was estimated to be \( >100 \) \( s^{-1} \) based upon the lack of curvature up to 50 \( \mu M \) actin. The slow phase was actin-independent and fairly constant throughout the actin concentrations tested. The slow phase is not an artifact coming from the MDCC-PBP system, because we did not observe the dual phase on skeletal myosin S-I (not shown). One possible explanation for the slow phase is "attached hydrolysis.
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The actin concentrations indicated are the final concentration. The fast dependence of the apparent rate constants of the fast and slow phase. The condition was the same as in inset. The standard error of the 3–5 independent experiments.

Actin Binding to M10IQ1—The rate of ADP binding to acto-M10IQ1 was measured by monitoring the change in the fluorescence intensity of Mant-ADP upon binding. In the absence of actin, the rate of Mant-ADP binding showed a hyperbolic dependence on the Mant-ADP concentration to yield the maximum rate of 1.11 ± 0.07 s⁻¹ (Fig. 7A). These results suggested that ADP binding is a two-step process. The first step is the formation of a collision complex and the second one is the conformational transition. The observed maximum rate constant represents the sum of the forward and backward steps, i.e. $k_3 + k_{-3}$ (Scheme 1), of the second step. The y-intercept of the Mant-ADP dependence gave 0.28 ± 0.06 s⁻¹, suggesting that the rate constant of Mant-ADP-off is 0.28 s⁻¹. The Mant-ADP off-rate was directly determined by measuring the decrease in Mant-ADP fluorescence intensity upon addition of 3 mM ATP to M10IQ1Mant-ADP (Fig. 7B). The rate constant of 0.27 s⁻¹ was obtained, which is consistent with the value obtained from Fig. 7A. The affinity of ADP for M10IQ1 ($K_{ADP}$) was calculated to be 1.2 μM.

In the presence of actin, the Mant-ADP binding rate increased with the Mant-ADP concentration to yield the second order rate constant of 2.6 ± 1.3 μM⁻¹ s⁻¹. The dissociation rate constant of 17 ± 3.6 s⁻¹ was obtained by extrapolation of the binding rate to zero Mant-ADP concentration (Fig. 8A). The rate of ADP dissociation was also directly measured by mixing acto-M10IQ1/ADP complex with excess ATP (Fig. 8B). The rate of the change in the light scattering of acto-M10IQ1 increased with ATP but unlike the result shown in Fig. 3, the rate was saturated at lower ATP concentrations to yield the maximum rate of 23 ± 2.0 s⁻¹. This is explained by the ADP dissociation step ($k_{-3}$) from acto-M10IQ1. The ADP dissociation rate constants obtained from the different experiments were in good agreement, hence assumed 23 s⁻¹ for $k_{-3}$. The affinity of acto-M10IQ1 for ADP was calculated from these results ($K_{ADP}$) was 8.8 ± 4.4 μM, a value consistent with the $K_{ADP}$ value of 6.4 μM obtained previously from the steady-state actin-activated ATPase measurement (16). The ADP dissociation rate was two times larger than the $V_{max}$ of the steady-state ATP hydrolysis cycle rate, indicating that this step is not the sole determinant of the cycle rate although it would contribute to the rate limitation of the ATP hydrolysis cycle to a certain extent. The rate of ADP dissociation was significantly increased by actin by ~100-fold.

Actin Binding to M10IQ1—The rate of actin binding to M10IQ1 was measured using pyrene-actin. The fluorescence intensity of pyrene actin was rapidly decreased upon the addition of M10IQ1 as a result of the binding of M10IQ1 to pyrene-actin whereas the fast phase remained at the same rate (~5.5 s⁻¹, not shown), suggesting the slow phase came from the ATP rebinding during the observation. With the given ATP concentration (0.23 μM), the rate constant of the forward reaction is ~1 s⁻¹. The rate constant of ~−1 s⁻¹ for the reverse reaction (that is possible with $k_{-2} = 0.2 ± 1.3$, Fig. 2) together with the equilibrium of ATP hydrolysis step ($K_{ATP} = 0.85$), explains the observed ~40% contribution of the slow phase to the total phase (Fig. 6A, inset). We think that the attached hydrolysis (AMT-AMDP) is negligible under the condition we employed in this study (see “Discussion”). We conclude that the phosphate release (>100 s⁻¹) is not the rate-limiting step of the acto-M10IQ1 ATPase cycle. We assume $k_{-4}$ and $k'_{-4}$ as ~0 s⁻¹, because 2 mM phosphate did not inhibit the ATPase activity of M10IQ1 (not shown).

**Fig. 6. Phosphate release from M10IQ1.** Phosphate release from M10IQ1 was monitored through MDCC-PBP fluorescence enhancement upon binding to phosphate released from M10IQ1 by double mixing experiment. 1 μM M10IQ1 was first mixed with 0.7 μM ATP, and aged for 5 s. Then the solution was mixed with various concentration of actin (0–150 μM). Because the volume ratio of the syringes was 1:1:1, the final concentrations were one-third of the originals. A, typical recording of the MDCC-PBP fluorescence enhancement at 0 μM actin. The fluorescence change was best fitted by single exponential, and the apparent rate was 0.06 s⁻¹. The inset shows MDCC-PBP fluorescence enhancement at 5 μM (in final) actin. The transient was best fitted by double exponential, and the apparent rates were 5.8 s⁻¹ and 0.4 s⁻¹. B, actin dependence of the apparent rate constants of the fast and slow phase. The actin concentrations indicated are the final concentrations. The fast phase was actin-dependent with the apparent second order rate constant of 0.71 μM⁻¹ s⁻¹, whereas the slow phase was actin-independent. The inset shows the apparent rate constants of the slow phase as a function of M10IQ1. The condition was the same as in A except 5 μM actin (in final) and various concentrations of M10IQ1 were employed. The x-axis indicates the final concentration (after mix) of M10IQ1. All solutions contained buffer B plus 0.2 mg/ml calmodulin, 0.01 units/ml FNPase, 0.3 mM EGTA, and 3 μM MDCC-PBP. The error bars represent the standard error of the 3–5 independent experiments.
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\begin{align*}
M + T & \overset{k_{-1}}{\underset{k_{-2}}{\rightleftharpoons}} (MT) \\
M + A & \overset{k_{+4}}{\underset{k_{-4}}{\rightleftharpoons}} AMD \\
M + D & \overset{k_{+5}}{\underset{k_{-5}}{\rightleftharpoons}} (MD) \\
AM + D & \overset{k_{+6}}{\underset{k_{-6}}{\rightleftharpoons}} AM + D \\
\end{align*}
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Scheme 1. Reaction scheme of myosin X ATPase. Abbreviations: \(A\), actin; \(M\), myosin; \(T\), ATP; \(D\), ADP; \(P\), phosphate.

actin (Fig. 9A, inset). The binding rate constant increased linearly with actin concentration to yield a second order rate constant of \(4.1 \pm 0.2 \, \text{mM}^{-1} \, \text{s}^{-1}\). The dissociation rate constant of \(1.4 \pm 0.2 \, \text{s}^{-1}\) was obtained from the y-intercept (Fig. 9A).

The dissociation rate constant of actin from acto-M10IQ1 was also measured by mixing an excess amount of non-labeled actin with the pyrene-acto-M10IQ1 complex. The fluorescence intensity of pyrene-actin was increased upon mixing that reflects the dissociation of pyrene-actin from acto-M10IQ1 complex (Fig. 9B). The observed rate constant of \(1.3 \pm 0.1 \, \text{s}^{-1}\) agrees well with the y-intercept of Fig. 9A. Based upon these results, \(K_{d} (K_{d1} \text{ in Scheme 1) of acto-M10IQ1 in the absence of nucleotides can be estimated to be 0.3 \, \text{mM}. This value is much higher than those of other known myosins indicating that the rigor binding of acto-myosin X is much weaker than those of other myosins.

The rate of actin binding to M10IQ1 was also measured in the presence of ADP using the same procedure. The time course of the change in pyrene fluorescence intensity was best fitted with double exponential kinetics (Fig. 10A, inset). Both phases showed linear actin concentration dependence. The second order rate constants of the fast and slow phases were \(1.3 \pm 0.1 \, \mu \text{M}^{-1} \, \text{s}^{-1}\) and \(0.13 \pm 0.02 \, \mu \text{M}^{-1} \, \text{s}^{-1}\), respectively (Fig. 10A). From the y-intercepts of the two phases, the rate constants for actin dissociation were estimated to be \(1.7 \pm 0.1 \, \text{s}^{-1}\) and \(0.12 \pm 0.02 \, \text{s}^{-1}\) for the fast and slow phases, respectively. The dissociation of actin from acto-M10IQ1/ADP was measured by monitoring the fluorescence change of pyrene-actin upon mixing pyrene-acto-M10IQ1/ADP with excess non-labeled actin (Fig. 10B). The increase in the pyrene fluorescence intensity followed single exponential kinetics and yielded the rate constant for actin dissociation of \(2.1 \, \text{s}^{-1}\). This value is consistent with the value obtained from the fast phase in Fig. 10A. The identity of the slow phase observed in Fig. 10A is unclear. Based upon these rate constants, the affinity of actin to M10IQ1/ADP was calculated to be \(1.6 \, \mu \text{M}\) that is significantly weaker than those of other unconventional myosins such as myosin IC (55 nM) (29), myosin Va (7.6 nM) (28), and myosin VI (47 nM) (27).

Consistently, a significant amount of M10IQ1 was recovered in the supernatant when M10IQ1 (1 \, \mu \text{M}) was co-precipitated with actin (5 \, \mu \text{M}) in the presence of ADP (Fig. 10B, inset). The fraction of M10IQ1 in the supernatant and pellet was determined by densitometry to be 22 and 78%, respectively. These values agree well with the calculated values, i.e. 27 and 73% in the supernatant and pellets, respectively, based upon the \(K_{d} = 1.6 \, \mu \text{M}\) estimated from the dissociation and binding rate constants.

Duty Ratio of Myosin X—The duty ratio (fraction of strong actin-bound myosin during ATPase cycle) can be estimated by comparing a \(K_{ATP}\) value obtained from ATPase assay at saturating actin (\(K_{m, \text{ATP}}\) of \(1 \, \mu \text{M}\)) with a \(K_{ATP}\) value obtained from ATP-dependent in vitro actin gliding assay (\(K_{m, \text{motility}}\)). This criterion has been employed to evaluate duty ratio of other motor proteins (31, 32). If we define the total time taken for completion of a single ATPase cycle at saturating ATP as \(T_{\text{total}}\), myosin spends \(T_{\text{total}} - T_{\text{motility}}\) waiting for next ATP binding at \(K_{m, \text{ATP}}\), where myosin takes \(2 \times T_{\text{total}}\) for each cycle (thus \(K_{ATP} = 2 \times T_{\text{total}}\)). Likewise, when we define attachment time of myosin to actin during ATPase cycle at saturating ATP as \(T_{\text{motility}}\), the ATP concentration at which a myosin spends \(T_{\text{motility}}\) is the ATP concentration at which a myosin spends \(T_{\text{total}} - T_{\text{motility}}\) waiting for next ATP binding (thus \(K_{ATP} = T_{\text{total}} - T_{\text{motility}}\)). Because the duty ratio is defined as \(T_{\text{motility}} / T_{\text{total}}\), the duty ratio can also be defined as \(K_{ATP} / K_{m, \text{motility}}\). We performed an ATP-dependent in vitro motility assay (Fig. 11A) and steady-state ATPase
assay at various actin concentrations (Fig. 11) on M10IQ1. The $K_m$ motility value obtained from this experiment was 6.1 ± 0.7 μM, which is consistent with the value previously obtained for the HMM-like myosin X construct (5.6 μM) (16). The $K_m$ ATPase is actin-dependent, and the maximal $K_m$ ATPase value was estimated to be 4.3 ± 0.5 μM. Therefore, we calculated the duty ratio of M10IQ1 as 0.70 ± 0.11.

**DISCUSSION**

**Overview**—In the present study, we analyzed the enzyme kinetic mechanism of the acto-myosin X ATP hydrolysis cycle. Based upon the obtained results, we concluded that myosin X is a high duty ratio motor. There are several unique features for the acto-myosin X ATPase reaction. First, the strong actin binding state (AMD) of myosin X is much weaker than other known myosins. The dissociation constant for the binding of myosin X/ADP to actin is 1.6 μM, which is significantly larger than those for myosin IC (55 nM), myosin Va (7.6 nM), and myosin VI (47 nM) (27–29). The result was also confirmed by actin co-sedimentation in the presence of ADP, in which a significant fraction of myosin X was recovered in the supernatant (Fig. 10B, inset). Second, the phosphate release step is not the rate-limiting step of acto-myosin X ATPase reaction despite its fast ADP release rate (2-fold larger than the $V_{max}$). Third, the

FIG. 8. The interaction of Mant-ADP with acto-M10IQ1. A, binding of Mant-ADP to acto-M10IQ1. The mixture of 1 μM M10IQ1 and 2 μM actin was mixed with various concentrations of Mant-ADP. The final concentrations are half of the originals. The change in the fluorescence intensity showed a single exponential increase, and the apparent rates ($k_{obs}$) were plotted against Mant-ADP concentration. The inset shows a typical recording of Mant-ADP fluorescence change at 2 μM Mant-ADP (in final). B, dissociation of ADP from acto-M10IQ1/ADP complex. The mixture of 0.5 μM M10IQ1, 1 μM actin, and 0.1 mM ADP was mixed with various concentrations of ATP, and the dissociation rate of acto-M10IQ1 was monitored by measuring the light scattering intensity. The time course of light scattering intensity was analyzed by single exponential fitting as in Fig. 3, and the apparent rates were plotted against ATP concentration. C, ADP release from pyrene-acto-M10IQ1. The mixture of 1 μM M10IQ1, 1 μM pyrene-actin, and 0.2 mM ADP was mixed with 1 mM ATP (before mix), and the change in pyrene-actin fluorescence intensity was monitored. The transient showed a double exponential kinetics to yield 503 s⁻¹ and 23.5 s⁻¹ with ~1:1 amplitude ratio. Conditions were the same as described in the legend to Fig. 2. The error bars represent standard error from 3–4 independent experiments.

FIG. 9. Actin binding to M10IQ1 in the absence of ADP. A, actin binding to M10IQ1. 0.25 μM M10IQ1 and various concentration of pyrene-actin were mixed and the decrease in the fluorescence signal because of the binding of the two proteins was monitored. The inset shows a typical recording of the experiment performed at 0.5 μM actin. The transients followed single exponential kinetics, and the apparent rate was plotted against pyrene-actin concentration. B, dissociation of actin from M10IQ1. The mixture of 1 μM M10IQ1 and 1 μM pyrene-actin was mixed with excess amount of non-labeled actin (40 μM before mix), and the increase in the fluorescence signal of pyrene-actin was monitored. The apparent rate of 1.3 s⁻¹ was obtained from single exponential analysis. Conditions were the same as described in the legend to Fig. 2. The error bars represent standard error from three independent experiments.
Kinetic Mechanism of Myosin X ATPase...

The Kinetic Mechanism of the Myosin X ATPase Cycle—All rate constants and equilibrium constants obtained in the present study are summarized in Table I. The overall ATPase rate of myosin X in the absence of actin is explained by the fast equilibria of AMDP. The apparent rate of 2.1 s⁻¹ was obtained from single exponential fitting. *Inset,* co-precipitation of M10IQ1 with actin in the presence of ADP. The mixture of 1 µM M10IQ1, 5 µM actin, and 0.1 mM ADP was subject to ultracentrifugation. The supernatant and pellets were subjected to SDS-PAGE followed by densitometry to quantitate the amount of proteins. 22% of M10IQ1 was found in the supernatant. Experimental conditions were the same as described in the legend to Fig. 2. The error bars represent standard error from three independent experiments.

...equilibrium of ATP hydrolysis step is significantly shifted to MT, and approximately equal fractions of MT and MDP are present in the ATPase cycle. This small burst size contributes to the overall ATPase rate of myosin X by reducing the fraction of AMDP.

The kinetic mechanism of myosin X ATPase is shown in bold in Scheme 1. The broken line in Fig. 1 shows a typical example of the experiment done at 1 µM (after mix) pyrene-actin. The fluorescence transients followed double exponential kinetics, and both the fast and the slow apparent rates were plotted against pyrene-actin concentration. B, dissociation of actin from M10IQ1. The mixture of 1 µM M10IQ1, 1 µM pyrene-actin, and 0.2 mM ADP (before mix) was mixed with excess amount of non-labeled actin (40 µM before mix) with 0.2 mM ADP, and the increase in the fluorescence signal of pyrene-actin was monitored. The apparent rate of 2.1 s⁻¹ was obtained from single exponential fitting. *Inset,* co-precipitation of M10IQ1 with actin in the presence of ADP. The mixture of 1 µM M10IQ1, 5 µM actin, and 0.1 mM ADP was subject to ultracentrifugation. The supernatant and pellets were subject to SDS-PAGE followed by densitometry to quantitate the amount of proteins. 22% of M10IQ1 was found in the supernatant. Experimental conditions were the same as described in the legend to Fig. 2. The error bars represent standard error from three independent experiments.

...rate constants and equilibrium constants obtained in the present study. The difference can be explained by the accuracy of the determination of protein (or active site) concentration. In our conclusions and their conclusions. First, the rate of the entire ATPase cycle obtained in this study is nearly three times larger than that reported by Kovacs et al. (30). The $V_{\text{max}}$ of HMM-like myosin X reported in our previous study was also smaller than the $V_{\text{max}}$ obtained in the present study. The difference can be explained by the accuracy of the determination of protein (or active site) concentration. In the present study, we directly determined the active site concentration by measuring the radioactive ADP incorporated into M10IQ1 in the presence of vanadate (see “Experimental Procedures”). This enabled us to determine the effective protein concentration much more accurately than with routine methods such as the dye binding method or biuret method. It should...
be noted that in the case of myosin X, the conventional active site titration method using ATP or Mant-ATP would not provide an accurate estimation because of the presence of a significant reverse reaction of the ATP binding step.

Second, they found a slow P1 off-phase from myosin X in the presence of actin and argued that this was caused by the “attached hydrolysis.” Based on our results, we think that the slow P1 off-phase is not derived from the attached hydrolysis, but because of the reverse reaction of the ATP binding step (see below and also “Results”).

**Attached Hydrolysis Pathway (AMT-AMDP)—Kovacs et al. (30)** suggested that the slow attached hydrolysis plays a significant role in the acto-myosin X ATPase cycle. This conclusion was based on the finding of a slow P1 off-phase in the P2 release kinetics of acto-myosin X. We also found the dual phase kinetics for phosphate release in the presence of actin, and the result was very similar (Fig. 6). However, because the slow rate was M10IQ1 concentration-dependent, we concluded that the slow phase is derived from ATP rebinding because of the reverse reaction (47 s⁻¹). Actually, our model explained the overall ATPase cycle very well without the slow attached hydrolysis (Fig. 1B, broken line).

Furthermore, the presence of the slow attached hydrolysis pathway in the ATPase cycle of myosin X is inconsistent with the results of actin gliding assay. We obtained a K₉₉₉₉₉₉₉ (motility) value of 6.1 μM for M10IQ1 (Fig. 11A). If T on is defined as the actin attached time of M10IQ1 during the ATPase cycle at a saturating ATP condition, the half-velocity of M10IQ1 can be explained by adding an additional T on time for ATP binding at the ATP concentration of K₉₉₉₉₉₉₉ (motility). The ATP binding rate at 6.1 μM ATP is 21 s⁻¹ (3.4 μΜ⁻¹ s⁻¹ × 6.1 μM), which is close to k₉₉₉₉₉₉₉ (23 s⁻¹) for acto-M10IQ1. Because all mysins involved in the translocation of actin filaments are under a saturating actin condition in an *in vitro* motility assay, if there is a slow S₉₉₉₉₉₉₉ (motility) value of 0.3 μM, which is inconsistent with the experimentally determined value of 6.1 μM.
Therefore, we propose that the attached hydrolysis pathway (AMT-AMDP) is negligible under the condition we employed in the present study. The \( k_{-2} \) of \( \sim 1 \text{ s}^{-1} \) would also affect the estimation of \( K_3 \) determined by the single turnover quenched-flow experiment, because the experiment also employed very low concentrations of ATP. The \( K_3 \) value of 0.85 was determined by assuming \( k_{-2} = -0 \text{ s}^{-1} \). If we assume \( k_{-2} = 1 \text{ s}^{-1} \) under the condition we measured the burst size (1 \( \mu M \) M10IQ1 and 0.5 \( \mu M \) ATP, Fig. 5A), \( -15\% \) of ATP remains unbound to M10IQ1. Thus the actual \( K_3 \) value, which should be estimated from the apparent burst size of 0.46 (Fig. 5A) will be 1.18, but not 0.85. However, this change does not influence our overall kinetic model.

**Processivity of Myosin X**—It has been suggested that the processive movement of the two-headed myosin requires a duty ratio of greater than 0.5. Actually, myosin Va, a well known processive myosin, has a duty ratio of \( >0.7 \). The duty ratio of myosin X obtained from the present study was 0.6–0.7, which would allow myosin X to be a processive motor. The duty ratio of myosin X is not as high as that of myosin Va, since the fraction of AMD intermediate is significantly reduced because of the unfavorable equilibrium of the hydrolysis step (\( K_3 = 0.85 \)) under the experimental condition in the present study (25 °C). However, as pointed out previously on myosin Va (33), the hydrolysis equilibrium (\( K_3 \)) may greatly increase at physiologic temperature (\( -37 \text{ °C} \)). Therefore, the duty ratio of myosin X at body temperature could be even higher than that determined in the present study.

High duty ratio is a necessary, but not sufficient requirement for processive movement of myosins. In our previous study, we concluded that myosin X is a nonprocessive motor since myosin X (HMM-like) did not support the actin gliding movement at a low surface density at which myosin Va (HMM-like) did, although myosin X was able to support the actin movement at the surface density much lower than myosin II. We assume that this was because our previously employed HMM-like myosin X containing the coiled-coil domain did not form a stable dimer. Myosin VI has been reported as a high duty ratio motor having ADP off as the predominant rate-limiting step. It is processive when it is dimer (34, 35); however, it becomes nonprocessive when it does not form a dimer (36). Although myosin X has been predicted as a dimer, because it contains a putative coiled-coil region, it might be a monomer as in the case of myosin VI. In fact, the putative coiled-coil region of myosin X is very similar to that of myosin VI in terms of poor hydrophobic seam and high proportion of charged residues, which would prevent the region from making a stable coiled-coil. Whether native myosin X is processive or not is an important question, and further studies are required to answer this question.

**How Is the Kinetic Mechanism of Myosin X Tuned Up for the Cellular Function?**—Recent studies have shown that myosin X transports cargos in cells (7). It was found that myosin X moves Mena/VASP from the root to the tip of filopodia (8). Myosin X also transports \( \beta \)-integrin to the tip of filopodia (7), suggesting that myosin X moves the transmembrane cargos to the final destination. It has been thought that the processive motor, having a high duty ratio, is suitable for cargo transportation. In the myosin superfamily, myosin Va has been shown to be a cargo transporting motor, which moves melanosomes in melanocytes, endoplasmic reticulum in the giant axons of the squid nervous system (37), and synaptic vesicles in brain (38–40).

Biocomplocal and biochemical studies have shown that myosin Va is a processive motor that travels on an actin filament for a long distance without dissociating from actin (10–13). Consistent with these findings, the kinetic analysis revealed that myosin Va is a high duty ratio myosin that spends more than 70% of the ATP hydrolysis cycle time in the strongly actin-bound form (28).

The present study revealed that the duty ratio of myosin X is higher than 50% suggesting that myosin X can be a processive motor. This is consistent with the view that myosin X functions as a cargo transporter based upon recent cell biological findings. Previously observed non-processive behavior of HMM-like myosin X constructs is likely caused by the unstable coiled-coil structure of myosin X.

How does myosin X move cargos for a significant distance in cells? One possibility is that native myosin X forms a two-headed structure and moves processively on actin filaments like myosin Va does. In this case, the tail domain helps form the dimer formation together with the weak coiled-coil domain. The second possibility is that multiple single-headed molecules attach to the cargo and concertedly transport the cargo. It has been suggested that a cluster of myosin I, a non-processive myosin, may be able to transport the cargos (41). The third possibility is that the dissociation of myosin X from the actin cable does not diffuse the cargo-myosin X complex away from the actin cable. Therefore, myosin X can re-bind to actin to move the cargo successively. It was shown that myosin X moves \( \beta \)-integrin, a transmembrane molecule, and it is anticipated that the lateral diffusion of the transmembrane molecules is much slower than the diffusion of cytosolic proteins. Furthermore, it is known that \( \beta \)-integrin forms a large multimolecule complex of molecular mass of much greater than 1,000 kDa. Based upon the present results, the time myosin X dissociated from actin is less than 30 ms, and it is plausible that the lateral diffusion of the molecular complex of greater than 1,000 kDa within this time is less than the head-neck length of myosin X. Therefore, it is expected that the cargo does not move away from the actin cable during the myosin X cross-bridge cycles, thus enabling myosin X to transport the membrane-bound cargos.

In summary, the present study revealed that myosin X is a high duty ratio actin-based motor, implying that myosin X is a suitable motor protein transporting cargos in cells. Physiological function and the nature of myosin X-based cargo transportation require further studies including the native structure and regulation of the myosin X molecule.

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