The Kinetic Mechanism of Mouse Myosin VIIA*

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Myosin VIIa is crucial in hearing and visual processes. We examined the kinetic and association properties of the baculovirus expressed, truncated mouse myosin VIIa construct containing the head, all 5IQ motifs and the putative coiled coil domain (myosin VIIa-5IQ). The construct appears to be monomeric as determined by analytical ultracentrifugation experiments, and only single headed molecules were detected by negative stain electron microscopy. The relatively high basal steady-state rate of 0.18 s⁻¹ is activated by actin only by ~3.5 fold resulting in a V_max of 0.7 s⁻¹ and a K_{ATP} of 11.5 μM. There is no single rate-limiting step of the ATP hydrolysis cycle. The ATP hydrolysis step (M-T == M-D-P) is slow (12 s⁻¹) and the equilibrium constant (K_D) of 1 suggests significant reversal of hydrolysis. In the presence of actin ADP dissociates with a rate constant of 1.2 s⁻¹. Phosphate dissociation is relatively fast (>12 s⁻¹), but the maximal rate could not be experimentally obtained at actin concentrations ± 50 μM because of the weak binding of the myosin VIIa-ADP-P complex to actin. At higher actin concentrations the rate of attached hydrolysis (0.4 s⁻¹) becomes significant and partially rate-limiting. Our findings suggest that the myosin VIIa is a “slow”, monomeric molecular motor with a duty ratio of 0.6.

Myosins are a large family of motor proteins with more than 35 classes (1). They are fundamental to numerous cellular functions such as cell motility, muscle contraction, cytokinesis, and intracellular transport (2). Myosins use chemical energy from ATP hydrolysis to perform motion and work in muscle and non-muscle cells by moving along the actin filaments. Conventional myosins (skeletal, cardiac, smooth) form bipolar filaments and have a role in force generation, whereas unconventional myosins usually work as single molecules or as an assembly of a few molecules in nonmuscle cells where they either have a role in generating force or transporting cargo.

The Class VII unconventional myosins are present in a wide range of organisms including human, mouse, zebrafish, Dicyostelium discoideum, and Drosophila. They are postulated to have a role in cell adhesion, endocytosis, cargo transport, and tension maintenance (3, 4, 5–7). Mammals and Drosophila have two isoforms VIIa and VIIb. Mammalian myosin VIIa is expressed in a variety of tissues including the inner ear, retina, lung, kidney, small intestine, and testis (8, 9). In the hair cells of the inner ear myosin VIIa is localized to the lateral and ankle links of stereocilia and serves in tension maintenance (10). It is also found in the pericuticular necklace where it might be involved in vesicular transport. In the retina myosin VIIa has a role in the opsin transport from the inner segment to the outer segment of the photoreceptors cells (11). In addition myosin VIIa is enriched in the apical actin-rich domain of the pigmented epithelium playing a role in distributing melanosomes. These functions require interaction with Rab27, a small GTPase, which binds to melanosome and to myosin VIIa via the linker proteins Slac2-c and MyRIP (12–14). This is similar to the manner that myosinV transports melanosome in melanocytes through the melanophilin-Rab27a complex (15).

The physiological significance of myosin VIIa functions is indicated by severe pathologies associated with mutations in the myosin VIIa gene. In human, myosin VIIa mutations cause a variety of sensory neuronal disorders including the Usher syndrome IB, characterized by congenital deafness, vestibular dysfunction and retinitis pigmentosa and two autosomal recessive hearing disorders, DFNA11 and DFNB2 (16, 17). In mice, myosin VIIa mutants lead to the shaker-1 phenotype displaying severe sensory dysfunction due to the disorganization of the stereocilial bundles in the auditory hair cells (18). Finally, myosin VIIa supports sensory functions in Drosophila and zebrafish suggesting an evolutionary conservation of its functions (7, 19).

The full-length myosin VIIa consists of the N-terminal motor domain followed by the neck region containing five IQ motifs where the calmodulin light chains bind and a tail domain which has two MyTH4 (myosin tail homology) domains separated by the SH3 (Src-homology) domain and two FERM (band 4.1/ezrin/radixin/moesin homology) domains, which link the myosin VIIa protein to other cytoskeletal proteins.

Structural, biochemical, and single molecule motility studies revealed that the Drosophila myosin VIIa is a monomeric, high duty ratio motor which can exhibit processive motion when artificially dimerized (20, 21). Although the domain structure of the mammalian and Drosophila myosin VIIa is the same, the motor domain sequence analysis shows that the fly myosin VIIa is in a different phylogenetic cluster from the mammalian myosin VIIa (22). It is therefore possible that the mammalian myosin VIIa has a different motor biochemistry.
Myosin VIIa Is a Slow, High Duty Ratio Motor

Steady-state ATPase activity and in vitro motility (23–25) have been measured for mammalian myosin VIIa; and recently several Usher-1B mutants of the human myosin VIIa have been shown to have reduced or hampered ATPase activity (26), but detailed mechanistic study has not been performed on this isoform. In the present study, we report the kinetic characterization of the recombinant wild type mouse myosin VIIa. Our findings suggest that this motor has a high duty ratio, which is consistent with its function in tension-bearing and possibly in cargo transport.

EXPERIMENTAL PROCEDURES

Expression and Purification of the Myosin VIIa Protein—A cDNA fragment encoding the first 934 amino acids of the mouse myosin VIIa gene (myosin head +5IQ+ putative coiled coil domain) and the Flag tag (DYKDDDDK) were subcloned into the Pfastbac1 vector (generously donated by Jennifer Fordham, King’s College London) (Fig. 1A). Recombinant bacmid DNA containing the myosin VIIa sequence (myosin VIIa-5IQ) was obtained by transforming the Pfastbac myosin VIIa cDNA into DH10Bac cells (Invitrogen). The bacmid DNA was then transfected into Sf9 insect cells to generate the myosin VIIa-5IQ virus. The amplified high titer myosin VIIa-5IQ baculovirus was co-infected with the calmodulin virus into Sf9 cells. The expressed protein was purified using FLAG affinity chromatography (27), and then concentrated and fractionated on a MonoQ ion exchange column with a linear gradient of 0.1–0.5 M NaCl containing 10 mM MOPS, 0.1 mM EGTA, 2 mM MgCl2 (pH 7.0). Where required, ATP and ADP were purchased from Sigma-Aldrich.

Analytical Ultracentrifugation Experiments—Sedimentation velocity experiments were carried out on a Beckman Instruments Optima XL-I analytical ultracentrifuge with Rayleigh optics. Apparent sedimentation coefficient distribution patterns were computed by the time derivative method using signal averaging (33, 34). Sedimentation boundaries were analyzed both by using time derivative g(s*) analysis using the SEDANAL software for the analysis of interacting and non-interacting systems (34). The myosin VIIa was dialyzed against its respective buffer and the dialysate was used for all dilutions and as a reference buffer. A value of 0.736 cc/g calculated from the amino acid sequence was used for the partial specific volume.

Steady-state Basal and Actin-activated ATPase Measurements—Steady-state ATPase activities were measured as described in Forgacs et al. (29). Except that phallolidin actin was prepared by centrifuging the actin with equimolar phallolidin at 30,000 rpm for 3.5 h at 4 °C. The pellet was resuspended in a buffer containing 10 mM MOPS (pH 7.0), 2 mM MgCl2, 0.15 mM EGTA, and 1 mM DTT. The ATPase activity of the blanks containing actin was subtracted from the actomyosin data.

Steady-state Actin Binding Measurements—Myosin VIIa-5IQ was cosedimented with increasing concentrations of actin in a buffer containing 10 mM MOPS (pH 7.0), 2 mM MgCl2, 25 mM KCl, 0.15 mM EGTA, 1 mM DTT, 2 mM ATP, and 2 μM exogenous calmodulin. The buffer also contained an ATP-regenerating system of 1 mM phosphoenolpyruvate and 200 units/ml pyruvate kinase (35). Centrifugation was carried out at 250,000 × g for 20 min at 20 °C to pellet the actomyosin complex. Supernatant was saved and the pellet was resuspended in the buffer with equal volumes to the supernatant. A 10-μl aliquot of both the supernatant and the pellet was run on a 4–20% SDS-PAGE gel. The myosin band was quantified by densitometry both in the pellet and supernatant. The affinity to actin (Kd) was determined by plotting the fraction of myosin present in the pellet against the actin concentrations and fitting it to a hyperbola: % myosin bound = 100 × [(actin) (Kd + [actin])].

Quench-flow Experiments—Chemical quench experiments were done as described in White et al. (36).

Stopped-flow Experiments—All stopped-flow measurements were done at 20 °C using an SF-2001 stopped-flow apparatus (KinTek Corp.) fitted with two 2 ml and one 5 ml syringe. In double mixing experiments myosin and ATP were mixed, allowed to incubate for the desired time and then mixed with actin to give 2/9 dilution of myosin VIIa and nucleotide and 5/9 dilution of actin in the flow cell. Single mixing experiment resulted in 2/7 dilution of myosin and 5/7 dilution of actin or nucleotide in the flow cell. The excitation light from a 75 watt xenon lamp was selected by using a 0.2 m monochromator (Photon Technology International, South Brunswick, NJ).

2The abbreviations used are: dmantATP, 3′-O-(N-methylanthraniloyl)-5′ deoxy-adenosine 5′-triphosphate; dmantADP, 3′-O-(N-methylanthraniloyl)-2′-deoxy-adenosine 5′-diphosphate; Pn, inorganic phosphate; MDCC-PBP, 7-diethylamino-3-((2-maleimidyl)ethylamino)carbonyl coumarin-labeled phosphate-binding protein.
DmantATP was excited at 360 nm and the emission was selected using a 400 nm long-pass filter. In the experiments where MDCC-PBP was used the excitation wavelength was 430 nm and the emitted light was selected using a 450 nm long-pass filter. Stocks of myosin VIIa were diluted from a buffer containing 300 mM KCl to obtain the desired protein concentration and a KCl concentration of 87.5 mM for single mixing and 112.5 mM for the double mixing phosphate release experiment. Light scattering was measured by using an excitation wavelength of 450 nm and a 400 nm long-pass filter. Stocks of myosin VIIa were diluted from a buffer containing 300 mM KCl to obtain the desired protein concentration and a KCl concentration of 87.5 mM for single mixing and 112.5 mM for the double mixing phosphate release experiment. Light scattering was measured by using an excitation wavelength of 450 nm and a 400 nm long-pass filter. Stocks of myosin VIIa were diluted from a buffer containing 300 mM KCl to obtain the desired protein concentration and a KCl concentration of 87.5 mM for single mixing and 112.5 mM for the double mixing phosphate release experiment. Light scattering was measured by using an excitation wavelength of 450 nm and a 400 nm long-pass filter. Stocks of myosin VIIa were diluted from a buffer containing 300 mM KCl to obtain the desired protein concentration and a KCl concentration of 87.5 mM for single mixing and 112.5 mM for the double mixing phosphate release experiment. Light scattering was measured by using an excitation wavelength of 450 nm and a 400 nm long-pass filter. Stocks of myosin VIIa were diluted from a buffer containing 300 mM KCl to obtain the desired protein concentration and a KCl concentration of 87.5 mM for single mixing and 112.5 mM for the double mixing phosphate release experiment. Light scattering was measured by using an excitation wavelength of 450 nm and a 400 nm long-pass filter. Stocks of myosin VIIa were diluted from a buffer containing 300 mM KCl to obtain the desired protein concentration and a KCl concentration of 87.5 mM for single mixing and 112.5 mM for the double mixing phosphate release experiment. Light scattering was measured by using an excitation wavelength of 450 nm and a 400 nm long-pass filter. Stocks of myosin VIIa were diluted from a buffer containing 300 mM KCl to obtain the desired protein concentration and a KCl concentration of 87.5 mM for single mixing and 112.5 mM for the double mixing phosphate release experiment. Light scattering was measured by using an excitation wavelength of 450 nm and a 400 nm long-pass filter. Stocks of myosin VIIa were diluted from a buffer containing 300 mM KCl to obtain the desired protein concentration and a KCl concentration of 87.5 mM for single mixing and 112.5 mM for the double mixing phosphate release experiment. Light scattering was measured by using an excitation wavelength of 450 nm and a 400 nm long-pass filter. Stocks of myosin VIIa were diluted from a buffer containing 300 mM KCl to obtain the desired protein concentration and a KCl concentration of 87.5 mM for single mixing and 112.5 mM for the double mixing phosphate release experiment. Light scattering was measured by using an excitation wavelength of 450 nm and a 400 nm long-pass filter. Stocks of myosin VIIa were diluted from a buffer containing 300 mM KCl to obtain the desired protein concentration and a KCl concentration of 87.5 mM for single mixing and 112.5 mM for the double mixing phosphate release experiment. Light scattering was measured by using an excitation wavelength of 450 nm and a 400 nm long-pass filter. Stocks of myosin VIIa were diluted from a buffer containing 300 mM KCl to obtain the desired protein concentration and a KCl concentration of 87.5 mM for single mixing and 112.5 mM for the double mixing phosphate release experiment. Light scattering was measured by using an excitation wavelength of 450 nm and a 400 nm long-pass filter. Stocks of myosin VIIa were diluted from a buffer containing 300 mM KCl to obtain the desired protein concentration and a KCl concentration of 87.5 mM for single mixing and 112.5 mM for the double mixing phosphate release experiment. Light scattering was measured by using an excitation wavelength of 450 nm and a 400 nm long-pass filter.
Myosin VIIa Is a Slow, High Duty Ratio Motor

When the experiment was carried out in a buffer with differing neck length. This indicates either weaker binding and negative stain electron microscopy showed molecules of the heavy chains of the purified myosin VIIa by densitometric analysis. ATPase of 16 mM KCl, but the ATPase is slightly higher. When 100 mM KCl added). The steady-state ATPase measured by the NADH-coupled assay.

**TABLE 1**

| Rate constants | Value             | Method          |
|----------------|-------------------|-----------------|
| K_{ATP} (μM)   | 10.5 ± 1.7        | NADH assay      |
| V_{max} (s⁻¹)  | 0.18 ± 0.02       | NADH assay      |
| kₐ (μM⁻¹s⁻¹)   | 0.7 ± 0.02        | NADH assay      |
| k₋ (μM⁻¹s⁻¹)   | 0.4 ± 0.02        | NADH assay      |
| k₋₋ (s⁻¹)      | 10.5 ± 1.7        | NADH assay      |
| k₋₋₋ (s⁻¹)     | 11.5 ± 2.9        | NADH assay      |

Steady-state actin binding kₐ (μM⁻¹s⁻¹) 10 ± 2.62 cosedimentation

**FIGURE 4.** Myosin VIIa-5IQ binding to actin filaments. Concentration dependence of the observed rate (k_{DA}) of myosin VIIa binding to phalloidin actin in the presence (□) and absence (○) of 20 μM ADP. Experimental conditions and final concentrations in the cell: 0.6 μM myosin VIIa-5IQ, 3 μM CaM, 1.5–7.2 μM actin, 25 mM KCl, 10 mM MOPS, 3 mM MgCl₂, 1 mM EGTA, pH 7.5, 20 °C. The data were fit into a linear equation and resulted in a slope, which is the second order rate constant for actin binding of kₐ = 4.7 ± 0.25 μM⁻¹s⁻¹ in the absence and k_{DA} = 2.6 ± 0.25 μM⁻¹s⁻¹ in the presence of ADP. Inset shows the observed change in light scattering upon binding of 2.5 μM actin to 0.6 μM myosin VIIa. The solid line through the data is the best fit to a double exponential equation: I(t) = 0.06e^{-7.9t} + 0.03e^{-8.3t} + C.

**TABLE 2**

| Summary of transient kinetic parameters of the myosin VIIa-5IQ | Value | Nucleotide | Method          |
|---------------------------------------------------------------|-------|------------|-----------------|
| ATP binding         | kₐ (μM⁻¹s⁻¹) | 10.5 ± 2.62 | NADH assay      |
| Hydrolysis          | kₐ + k₋₋₋ (s⁻¹) | 12 ± 0.9 | ATP | SF Trypsin fluorescence |
| ADP binding         | k₋₋₋ (μM⁻¹s⁻¹) | 1.2 ± 0.66 | d mantATP | Single turnover SF |
| Actin binding       | k₋₋₋₋ (μM⁻¹s⁻¹) | 1.2 ± 0.26 | d mantATP | Single turnover SF |

exponential equations. The fast phase depends linearly on the actin concentration (Fig. 4). The apparent second order rate constant for myosin VIIa-5IQ binding to actin obtained from the slope is kₐ = 4.7 ± 0.25 μM⁻¹s⁻¹ in the absence and k_{DA} = 2.6 ± 0.25 μM⁻¹s⁻¹ in the presence of ADP (Table 2).

Single and Multiple Turnover Measurements—We have done single and multiple turnover stopped-flow measurements in which we rapidly mixed myosin VIIa-5IQ with different con-
fitting the data in Fig. 5 is similar to what was measured by the rate-limiting step of the basal dmantATPase measured by the fluorescence increase was fit well by a one exponential equation of dmantATP binding on the dmantATP concentration. The concentration of the active sites was 0.9 μM (96% theoretical based on A280). There is a 81% fluorescence increase by dmantATP binding to myosin VIIa. The same fluorescence increase is assumed for the ATP, MADP, and M-ADP-P, complexes. We attributed the initial phase of 1 s rapid increase in fluorescence followed by a slower decrease in concentrations of dmantATP in the absence of actin (37) (Fig. 5). Global fits of the data to Equation 1 were used to determine the rate constants of dmantATP binding, $k_T = 2.3 \mu M^{-1} s^{-1}$, the rate-limiting step of steady- state dmantATP hydrolysis, $k_{-DP} = 0.23 s^{-1}$, the affinity for ADP $K_{DP} = 3.5 \mu M$ and the concentration of the active sites, 0.96 μM measured by $A_{280}$. The rate-limiting step of the basal dmantATPase measured by fitting the data in Fig. 5 is similar to what was measured by the NADH–coupled assay with ATP in Fig. 3.

$$k_T \quad K_{AT} \quad K_{AP} \quad K_{DP} \quad K_{AD}$$

SCHEME 1. Positive subscripts denote the binding of the last ligand in the subscript, negative subscripts denote dissociation (e.g. $k_{-AD}$ is the dissociation of ADP from actomyosin VIIaADP). Abbreviations: A, actin; M, myosin VIIa-5IQ; T, ATP; D, ADP; P, phosphate.

ATP Binding to Myosin VIIa and Actomyosin VIIa—Myosin VIIa or actomyosin VIIa were mixed with dmantATP substrate. (The kinetic scheme for the myosin VIIa ATP hydrolysis cycle is depicted in Scheme 1.) Fig. 6 shows the dependence of the rate of dmantATP binding on the dmantATP concentration. The fluorescence increase was fit well by a one exponential equation and the rate constants for myosin and actomyosin VIIa were plotted against the dmantATP concentrations. A linear fit to the data resulted in an apparent second order binding rate constant ($k_T$) of 2.5 μM$^{-1}$ s$^{-1}$ for myosin VIIa binding to ATP. In the presence of actin the rate of dmantATP binding was reduced to 0.53 μM$^{-1}$ s$^{-1}$ ($k_{AT}$).

ATP Binding Measured by the Enhancement of the Intrinsic Tryptophan Fluorescence Intensity—Similar to several other myosins (e.g. myosin II and V), mouse myosin VIIa has a tryptophan residue (Trp$^{484}$) in its relay loop which acts as an intrinsic fluorescence sensor of conformational changes occurring in the nucleotide binding pocket resulting from the conformational change required for ATP hydrolysis (29, 38, 39, 40, 41). We carried out single-mixing stopped-flow fluorescence measurements by mixing myosin VIIa (treated with apyrase to ensure that the myosin VIIa has no ADP bound) with increasing concentrations of ATP. When substoichiometric ATP concentrations were used we observed two distinct phases; an initial rapid increase in fluorescence followed by a slower decrease in fluorescence. We attributed the initial phase of 1 s$^{-1}$ to ATP binding. The second phase of 0.24 s$^{-1}$ is consistent with the basal steady-state rate measured in the NADH coupled experiments and the rate of basal P$_i$ release (see below) (Fig. 7A). The dependence of the rate of fluorescence increase upon ATP concentration were fit to a hyperbolic equation with a maximal rate of 12 s$^{-1}$ and a $K_{ATP}$ of 5.4 μM (Fig. 7B). The maximal rate may be limited by the apparent hydrolysis rate constant ($k_{ATP} + k_{-ATP}$). The initial slope of $2.2 \mu M^{-1} s^{-1}$ (Table 2) is the second order rate constant for ATP binding which compares well with the values obtained from the dmantATP binding and the single turnover measurements. An alternative interpretation of the data that the tryptophan signal measures a conformational change that occurs prior to the hydrolysis step as proposed by Málnás et al. (42) is equally possible (see Equation 2 below). We opted for the simpler mechanism containing fewer intermediates because the conclusions with regarding to the function of
Myosin VIIa Is a Slow, High Duty Ratio Motor

FIGURE 7. ATP binding to myosin VIIa-5IQ measured by tryptophan fluorescence. A, 1 μM myosin VIIa was mixed in the stopped-flow cell with 0.7 μM ATP (1:1 stoichiometric). Experimental conditions: 10 mM MOPS, 3 mM MgCl₂, 25 mM KCl, 1 mM EGTA, pH 7.5, 20 °C. Three traces were averaged and fit to a two exponential equation with k⁺ = 1 s⁻¹ and k⁻ = 0.24 s⁻¹. B, 0.86 μM myosin VIIa was mixed with increasing concentrations of ATP (final conc.: 3.6 μM-142.8 μM). Experimental conditions were otherwise as in A. The traces from three experiments were averaged and fit to a one exponential equation. A fit of the data to a hyperbola (kobs = kmax/(1 + [ATP]/K₅₅) yielded a maximum rate constant of kmax of 11.5 ± 0.83 s⁻¹ and a K₅₅ of 5.4 ± 1.8 μM.

FIGURE 8. ATP hydrolysis measured by quenched-flow. Myosin VIIa-5IQ was mixed with ATP and allowed to react for the indicated amount of time and then quenched in acid. Experimental conditions: 50 mM KCl, 10 mM MOPS, 3 mM MgCl₂, 1 mM EGTA, pH 7.5, 20 °C. Final concentrations in the delay line were 1 μM myosin VIIa and 40 μM ATP (B). The solid lines through the data fit best to a double exponential equations (a) 36/77 e⁻¹.¹⁺ 41/77 e⁻¹.²² for (O) and (b) 10/58 e⁻⁰.₅⁺ 71/82 e⁻⁰.₇⁵ (O). A single exponential of I(t) = 1 e⁻¹.²² was the best fit for (B).

was also obtained by the stopped flow single turnover measurements carried out with dmantATP. The ratio of the fast and slow phase amplitudes (Af/Fs) is ~1, provides a measure of the equilibrium constant, K₅₅, of the hydrolysis indicating that there is significant reverse hydrolysis. In the experiments with higher [ATP] > [S] (1) (10 μM myosin VIIa and 40 μM ATP) a double exponential fit gave a rate of 6 s⁻¹ for the fast phase, which measured the rate of the hydrolysis step and 0.06 s⁻¹ for the slow phase which is consistent with a the steady-state rate of 0.24 s⁻¹. Combining the rate of hydrolysis at saturating ATP concentrations obtained from the stopped-flow tryptophan fluorescence experiments with the equilibrium constant, K₅₅ obtained from quenched-flow experiments provides an estimate of rates of ~6 s⁻¹ in both forward and reverse directions.

ADP Binding and Dissociation in the Absence and Presence of Actin—We measured the rate of dmantADP binding (k⁺, k⁻) by mixing myosin VIIa-5IQ or actomyosin VIIa-5IQ with increasing concentrations of dmantATP (Fig. 9, B and D). Time courses followed single exponentials (Fig. 9, A and C) and a linear fit of the observed rates resulted in a second order rate constant of 1.2 μM⁻¹ s⁻¹ for dmantADP binding to myosin VIIa-5IQ and 1.5 μM⁻¹ s⁻¹ to actomyosin VIIa-5IQ. The rate of dmantADP dissociation was obtained by premixing myosin (2.1 s⁻¹) or actomyosin VIIa-5IQ (1.7 s⁻¹) with dmantADP and chasing with ATP in the single-mixing stopped-flow (horizontal symbol on plots; data not shown).

Dissociation of Actomyosin and ActomyosinADP by ATP—The rate of dissociation of myosin VIIa (Fig. 10A) and myosin VIIaADP (Fig. 10C) from actin in the presence of ATP was measured by light scattering. Actomyosin was mixed with increasing concentrations of ATP. Time courses were monophasic. The observed rates were fast and showed a hyperbolic dependence on the ATP concentration with a maximal rate of 350 s⁻¹, and a K₅₅ of 295 μM (Fig. 10B). In the presence of ADP the observed maximal rate was 1.28 s⁻¹, which is significantly lower, because the dissociation of myosin from actin is limited by the rate of ADP release (k⁻) (Fig. 10D). The maximal rate of dmantADP dissociation from actomyosin VIIa was also measured by the decrease in light scattering observed after

the mouse myosin VIIa, which depends upon the rate-limiting step and duty ratio are the same.

M + ATP ⇄ M-ATP ⇄ M-ATP* ⇄ M*-ADP-P⁺ ⇄ M-ADP ⇄ M

( Eq. 2)

Quenched Flow Measurements of Presteady-State ATP Hydrolysis—To monitor ATP hydrolysis directly in the presteady-state we performed quenched flow experiments in the absence of actin (Fig. 8). When myosin VIIa-5IQ was rapidly mixed with substoichiometric amounts of [³²P]ATP under single turnover conditions (O and ), the time courses were fit to either a one exponential ( ) or two exponential equations (O). The two exponential fit resulted in a fast phase, which was limited by the rate of ATP binding and a slow phase (0.21 s⁻¹), which is essentially the same as the basal steady-state rate or the rate of phosphate release in the absence of actin. A similar rate
mixing actomyosin VIIa-dmantADP with 1 mM ATP in a single-mixing stopped flow. The change in light scattering was monophasic and resulted in a rate of 1.8 s⁻¹ for dmantADP dissociation (data not shown), which is in good agreement with the data obtained in the experiment in Figs. 9D and 10D.

**Phosphate Release**—We used a fluorescently labeled phosphate-binding protein (MDCC-PBP) to monitor Pi dissociation from the actomyosin VIIa-5IQ-ADP-Pi complex. In a double-mixing stopped-flow experiment, myosin VIIa was first mixed with ATP under single turnover conditions and incubated for 2 s to allow ATP binding and hydrolysis to occur, followed by mixing with actin to measure the rate of phosphate dissociation. The MDCC-PBP was present at the same concentration in all three syringes to obtain a large excess over phosphate in the stopped-flow cell. To prevent phosphate contamination, all solutions were preincubated with phosphate mop (for the components and concentrations see “Experimental Procedures”). Time courses were biphasic with equal fractional amplitudes (Fig. 11A) which correlates well with the equilibrium constant \(K_{\text{AD}}\) of 1 obtained for the hydrolysis. The slower phases did not show actin dependence and varied between 0.28 – 0.42 s⁻¹. We think that these rates measure the rate of actin-attached hydrolysis as was previously observed by White et al. (36). The observed rate constants for the fast phase exhibited a linear dependence on actin concentrations resulting in a slope \((K_{\text{AD}}/K_{\text{DPA}})\) of 0.05 μM⁻¹ s⁻¹ indicating that the affinity of the myosinADP-Pi complex to actin is weak (Fig. 11B). Kinetic simulation enabled us to obtain limits for the rate of phosphate dissociation, a \(k_{-\text{DAP}}\) of 12 s⁻¹ and a \(K_{\text{act}}\) of 200 μM (Fig. 11B). Significantly higher [actin] (10×) is required to reach the maximum rate of phosphate release than the steady-state rate because phosphate dissociation is not rate-limiting and the steady-state rate is mostly dependent on the rate of ADP release from the actomyosinADP complex.

**DISCUSSION**

**The Kinetic Mechanism of the Mouse Myosin VIIa ATP Hydrolysis**—All the rate constants and equilibrium constants obtained in the present study are summarized in Tables 1 and 2. The rate-limiting step of the basal ATPase is the rate of phosphate release. In the presence of actin the steady-state ATPase rate is activated ~3.5-fold. Maximal ATPase rates could only be obtained by the addition of exogenous calmodulin, since it appears that calmodulin readily dissociates from the heavy
chain. Our kinetic analysis indicates that there is no single predominant rate-limiting step of the actin-activated ATPase. The slowest step in the cycle is the ADP release (k_{AD}P) from actomyosin VIIa which has a rate of 1.2 s^{-1} and the attached hydrolysis at higher actin (0.4 s^{-1}). The maximal rate of the hydrolysis (k_{H+} + k_{AD}P) is 12 s^{-1} measured by tryptophan fluorescence. The equilibrium constant of the hydrolysis is \( k_{H+}/k_{AD}P = 1 \) indicating that the rate of forward hydrolysis is equal to the rate of reversal hydrolysis. The rate of P_i dissociation increases linearly with [actin] to 2.4 s^{-1} at 50 \( \mu \)M actin. Simulation indicates that a rate constant of \( k_{-AD}P \geq 12 s^{-1} \) is necessary to produce the observed linear dependence. At actin concentrations \( \geq 200 \mu \)M the rate of phospho-
Myosin VIIa Is a Slow, High Duty Ratio Motor

Comparison of the Mouse and Drosophila Myosin VIIa ATPase cycle—The kinetic mechanism of myosin VIIa shows differences between the mouse and Drosophila isomers. This is not surprising given that there is only a 60% sequence identity between mouse myosin VIIa-5IQ and the corresponding sequence in Drosophila. Both are high duty ratio motors. ADP dissociation from actomyosin VIIa is rate-limiting for the Drosophila myosin VIIa (43, 44). In mouse actomyosin VIIa, ATPase is limited by both the ADP release and the slow attached hydrolysis. The hydrolysis step (M-ATP $\rightarrow$ M-ADP-P) is relatively slow and reversible ($K_{eq}$ = 1 and $k_{H} + k_{H^{-1}}$ $\sim$ 12 s$^{-1}$) for both the Drosophila and the mouse myosin VIIa. $P_{i}$ dissociation from actomyosin-ADP-P$_{i}$ is fast for both myosins; $>$12 s$^{-1}$ (mouse) and $>$50 s$^{-1}$ (Drosophila) but the Drosophila myosin VIIa-ADP-P$_{i}$ has a 10-fold higher affinity to actin than the mouse. Mouse myosin VIIa has a high basal steady-state activity which is activated 3.5-fold by actin. In contrast the Drosophila motor has a lower basal steady-state rate that is activated $\sim$35-fold by actin.

Myosin VIIa Exhibits a High Duty Ratio—A high duty ratio means that the motor is in the actin attached state for the majority of its ATP cycle. The duty ratio of myosin VII-5IQ was calculated using the Scientist software to solve the differential equations for Scheme 1. At lower actin concentrations (25 mM) the duty ratio was low (0.42). At actin concentrations $\geq$ 200 $\mu$M myosin VIIa has a duty ratio of 0.6 (Fig. 12). The high duty ratio may occur in the cell by the localization of the myosin VIIa protein to regions of high actin concentrations (the estimated actin concentration in the stereocilia is 2–4 mM). A high duty ratio is necessary but not sufficient for a motor to be a processive cargo transporter. For a single motor to be processive the formation of dimers is necessary to enable walking with multiple successive steps along actin filaments (45, 46). However, myosin VIIa has a short putative coiled coil with a relatively high percentage of charged residues which suggests the formation of single $\alpha$-helix rather than a coiled coil structure. This is the case for myosin X, a monomeric motor which was found to have a single $\alpha$-helix following the IQ domains, extending its lever (47). A single $\alpha$-helical structure was also found for the Drosophila myosin VIIa (20, 21). Analytical ultracentrifugation experiments suggest that the truncated version of myosin VIIa utilized in this study has a sedimentation value of 5.92S and it appears to be a monomer with a molecular mass of $\sim$130 kDa. This molecular mass probably represents the heavy chain with one molecule of calmodulin bound (data not shown). Electron microscopy images obtained by negative staining using myosin concentrations similar to those in vivo (nanomolar range) also yielded only monomeric molecules. Additional images need to be collected to determine the precise structure of the truncated and the full length mouse myosin VIIa molecule, but it seems unlikely that the predicted coiled coil would enable dimerization of myosin VIIa in vivo. It is plausible that dimerization may occur in the cell via cargo binding as it was shown for myosin VI which forms dimers in the presence of adaptor proteins e.g. optineurin (48).

Kinetic Adaptation for Cellular Function—As described in the Introduction there are numerous cell biological studies which suggested that myosin VIIa functions both as a force generator and a cargo transporter. In the stereocilia myosin VIIa localizes to the actin bundles and forms the lateral and ankle links in between stereocilia along with its numerous binding partners e.g. vezatin and harmonin b and the cadherin/$\alpha$-catenin complex (49, 50). It has been suggested that myosin VIIa is responsible for maintaining the tension by linking the stereociliary membrane to the actin core thus causing tension in membrane-bound elements such as transducer channels, the tip link and the lateral links. Kros et al. observed that in the
Myosin VIIa Is a Slow, High Duty Ratio Motor

resting hair cells of myosin VIIa mutant mice, there is reduced hair bundle stiffness which could be due to the lack of tension on the lateral links. The study concludes that myosin VIIa could be an adaptation motor in hearing, because despite most aspects of the transduction being normal in these mice, the loss of force generator makes them deaf (51, 52). Myosin VIIa is also present in the cuticular plate where it connects the stereocilium to the actin rootlets in conjunction with harmonin thus contributing to the stability of the stereocilium. In the retina myosin VIIa has a role in opsin and melanosome transport. How myosin VIIa is able to transport cargo is still a question. Probably several monomeric myosin VIIa motors work together in ensembles or they function as single molecule dimeric cargo transporters. As mentioned above in vivo dimerization by cargo binding was demonstrated for myosinVI, so we speculate that similarly myosin VIIa could bind MyRip for example and transport the MyRip/melanosome/Rab27a complex in the cell.

In summary we found that the truncated mouse myosin VIIa is a slow motor with a relatively high duty ratio. It appears to be monomeric by electron microscopy, which is consistent with the presence of a single α-helix following the neck domain. These properties make this motor suitable to exert its function as a force sensor/tension maintainer in the stereocilia and as a putative cargo transporter in the retina.

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REFERENCES

1. Odrontiz, F., and Kollmar, M. (2007) Genome Biol. 8, R196
2. Mooseker, M., Forth B. (2008) in Myosins: A Superfamily of Molecular Motors. (Collucio, L. M., ed) pp.1–34, Springer, The Netherlands
3. Titus, M. A. (1999) Curr. Biol. 9, 1297–1303
4. Todi, S. V., Franke, J. D., Chee, M. K., Montague, R. A., Chen, T. L., Toyoda, H., and Brown, S. D. (1997) Nat. Genet. 16, 188–190
5. Gibson, F., Walsh, J., Mburu, P., Varela, A., Brown, K. A., Antonio, M., Beisel, K. W., Steel, K. P., and Brown, S. D. (1995) Nature 374, 62–64
6. Todi, S. V., Sivan-Loukianova, E., Jacobs, J. S., Kiehart, D. P., and Eberl, D. F. (2008) PLoS One 3, e2115
7. Yang, Y., Baboolal, T. G., Sthithananadan, V., Chen, M., Walker, M. L., Knight, P. J., Peckham, M., and Sellers, J. R. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 4189–4194
8. Umeki, N., Jung, H. S., Watanabe, S., Sakai, T., Li, X. D., Ikebe, R., Craig, R., and Ikebe, M. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 8483–8488
9. Yamashita, R. A., Sellers, J. R., and Anderson, J. B. (2000) J. Muscle Res. Cell Motil. 21, 491–505
10. Inoue, A., and Ikebe, M. (2003) J. Biol. Chem. 278, 5478–5487
11. Uudovichenko, I. P., Gibbs, D., and Williams, D. S. (2002) J Cell Sci. 115( Pt 2), 445–450
12. Al-Anari, A., Schonn, J. S., Kussel-Andermann, P., Blanchard, S., Des-