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Effect of ADP and Ionic Strength on the Kinetic and Motile Properties of Recombinant Mouse Myosin V*

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Mouse myosin V is a two-headed unconventional myosin with an extended neck that binds six calmodulins. Double-headed (heavy meromyosin-like) and single-headed (subfragment 1-like) fragments of mouse myosin V were expressed in Sf9 cells, and intact myosin V was purified from mouse brain. The actin-activated MgATPase of the tissue-purified myosin V, and its expressed fragments had a high V_{max} and a low K_{ATPase}. Calcium regulated the MgATPase of intact myosin V but not of the fragments. Both the MgATPase activity and the *in vitro* motility were remarkably insensitive to ionic strength. Myosin V and its fragments translocated actin at very low myosin surface densities. ADP markedly inhibited the actin-activated MgATPase activity and the *in vitro* motility. ADP dissociated from myosin V subfragment 1 at a rate of about 11.5 s^{-1} under conditions where the V_{max} was 3.3 s^{-1}, indicating that, although not totally rate-limiting, ADP dissociation was close to the rate-limiting step. The high affinity for actin and the slow rate of ADP release helps the myosin head to remain attached to actin for a large fraction of each ATPase cycle and allows actin filaments to be moved by only a few myosin V molecules *in vitro*.

Fifteen classes of myosins have been defined based on sequence analysis of the conserved motor domain of the molecule (1, 2). There has been extensive biochemical characterization of myosins from class I and II (1). In addition, some of the *in vitro* properties of chicken brain myosin V have been reported (3, 4). Myosin V is a two-headed molecule composed of two heavy chains weighing 212 kDa. Each heavy chain contains a typical myosin motor domain with a neck domain consisting of six IQ motifs that interact with myosin light chain subunits and calmodulin. Following this is a tail domain that contains regions of coiled-coil interspersed with nonhelical regions. Chicken myosin V copurifies with both calmodulin and essential light chain (5), but the stoichiometry of the various light chains has not been rigorously determined (5). The elongated appearance of the myosin head in rotary shadowed electron micrographic images, however, is consistent with a neck region that has many light chain subunits (4). In addition, chicken brain myosin V contains another low molecular mass subunit (8 kDa) termed the "dynein light chain," which has also been found in association with several other proteins including dynein and nitric-oxide synthase (6).

Myosin V genes have been found in humans, rats, mice, chickens, *Drosophila, Caenorhabditis elegans*, and *Saccharomyces cerevisiae* (4, 7–13). There are two myosin V genes in yeast and at least two genes for myosin V in mammals, termed Va and Vb. Mutations in yeast and mouse myosin V genes reveal that this myosin likely functions in vesicle transport (see Ref. 2 for review). The *dilute* gene in mouse encodes myosin Va (9). *dilute* mutations are characterized by pale coat color, and the severe alleles also display neurological defects. The coat color phenotype is due to defective trafficking of the melanosomes in melanocytes (14–17), whereas the neurological defects may be caused by alterations in synaptic transmission (18). Mutations in human myosin Va gene are responsible for Griscelli's syndrome, which has similarities to the phenotypes exhibited by *dilute* mice (19).

Chicken myosin V has an actin-activated MgATPase activity that is markedly activated by calcium and has a high apparent affinity for actin as measured by the amount of actin required for half-maximal activation of the MgATPase activity (3, 4). Myosin V, when bound to a nitrocellulose-coated surface, moves actin filaments in the *in vitro* motility assay. A recent study provides strong evidence that a single myosin V molecule can act processively to translocate an actin filament (20). This type of motility requires that the myosin head remains attached to actin for a large fraction of each ATPase cycle (*i.e.* has a high duty ratio) and has a high affinity for actin.

We have expressed single-headed, subfragment 1 (S1)[1]-like and double-headed, heavy meromyosin-like (HMM) truncated fragments of mouse myosin V and have characterized the MgATPase activity and *in vitro* motility activity of these recombinant fragments. We found that the kinetic behavior of mouse myosin V is very different from that of conventional myosin II proteins and is altered so that cellular vesicles could be moved by just a few myosin V molecules. In addition, we have purified mouse brain myosin V and compared some of its biochemical properties to those of the recombinant fragments.

MATERIALS AND METHODS

Construction of Mouse Myosin V HMM-like Fragment into Baculovirus Transfer Vector—A full-length cDNA clone of mouse myosin Va[2] was cut at a 5′ *Not*I prior to the ATG starting codon and a 3′ *Eco*47III site to give a fragment that encodes for 1091 amino acids corresponding to an HMM-like fragment. This fragment was subsequently linked to a FLAG epitope tag (DYKDDDDK or GACTACAAGGACGACGATGATAAG) followed by a stop codon and *Sma*I site at its 3′-end and

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[1] The abbreviations used are: S1, subfragment-1; HMM, heavy meromyosin; MOPS, 4-morpholinepropanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol.

[2] J. A. Hammer, III, unpublished data.

subcloned into pVL1392 (Invitrogen), a baculovirus transfer vector, between the *Not*I and *Sma*I sites.

Construction of Mouse Myosin V S1-like Fragment into Baculovirus Transfer Vector—Using the above FLAG-tagged mouse V as a polymerase chain reaction template, a DNA fragment of about 900 base pairs containing the unique *Sfi*I site at 1910 base pairs and 3′-ending at 2761 base pairs with the FLAG tag followed by a stop codon and *Sma*I site was generated. Substituting this polymerase chain reaction product into the above mouse myosin V HMM-like fragment in pVL1392 between sites *Sfi*I and *Sma*I produced the desired mouse myosin V S1-like fragment of 907 amino acid residues for baculovirus expression.

Preparation of Recombinant Proteins—Baculoviruses were produced in a manner similar to that described by Pato *et al.* (21). Briefly, the baculotransfer vector containing the desired cDNA was transfected into Sf9 cells aided by BaculoGold DNA (PharMingen). The resulting baculovirus was plaque purified and amplified to a titer of 5×10^8 to 2×10^{10} plaque-forming units/ml, and stored at 4 °C as a stock for further use.

Sf9 cells (about 1 liter of culture) were coinfected by the amplified heavy chain virus along with recombinant baculoviruses expressing calmodulin (a generous gift from Dr. Craig Montel, Johns Hopkins Medical School) and myosin essential light chain (LC17b) (22) for 3 days. After collection, the cell pellets were quickly frozen in liquid nitrogen and either used directly or stored at −80 °C. Thawed cell pellets were extracted with 10 mM MOPS (pH 7.0), 0.2 M NaCl, 2 mM ATP, 10 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mg/liter leupeptin, 1 mM dithiothreitol (DTT) and homogenized in a ground glass homogenizer. The mixture was stirred continuously in 4 °C for 20–30 min and sedimented at 47,000 × *g* for 10 min. The supernatant was mixed with Anti-FLAG M2 Affinity Gel (Sigma) and gently rocked for 1 h at 4 °C to ensure the binding of the FLAG-tagged myosin V to the affinity gel. This mixture was then sedimented at 1500 × *g* for 5 min. The gel pellet was washed once in buffer A (0.5 M NaCl, 10 mM MOPS (pH 7.0), 0.1 mM EGTA, 1 mM DTT, 0.1 mM PMSF) and once with buffer A plus 1 mM ATP and 5 mM $MgCl_2$ followed by another wash in buffer A alone. The gel was then washed with buffer B (10 mM MOPS (pH 7.0), 0.1 mM EGTA, 1 mM DTT, 0.1 mM PMSF) containing 1 mM EDTA followed by buffer A plus 1 mM EDTA to remove possible bound nucleotide. A final round of washes with buffer B followed by buffer A was carried out. The low ionic strength washes helps to remove a FLAG antibody cross-reactive protein of about 110 kDa. The gel was then resuspended in buffer A and packed into a column and washed further with buffer A. The FLAG-tagged myosin V HMM or S1 was eluted with buffer A containing 0.3 mg/ml FLAG peptide. The eluted fractions were analyzed by electrophoresis on a 15% SDS-polyacrylamide gel electrophoresis gel. The fractions containing myosin fragments were pooled and concentrated by precipitation with 60% $(NH_4)_2SO_4$ followed by sedimentation at 47,000 × *g* for 10 min. The pellet was solubilized in 1–5 ml of buffer B (200 mM KCl, 10 mM MOPS (pH7.0), 0.1 mM EGTA, 1 mM DTT, 0.1 mM PMSF) and dialyzed in 1 liter of the same buffer overnight with two changes to remove residual ammonium sulfate and ATP. The protein concentration was determined by the Bio-Rad Protein Assay using chicken gizzard smooth muscle myosin HMM as standard for the V HMM and smooth muscle myosin S1 for the V S1. Typically, the purified protein concentration was in the range of 1 mg/ml and was used within 3 days of purification or flash frozen and stored in liquid nitrogen for future use.

Purification of Mouse Brain Myosin V—Fresh mouse brains (40–80 g) were used as the starting material. The preparation was essentially according to Cheney (23) with the exception that an 0.9 × 90-cm S500 column was used, and the myosin V was eluted from the trimethylaminoethyl Fractogel 650-S column (EM Separations, Gibbstown, NJ) by an 0.5 M KCl, 20 mM HEPES (pH 7.5), 1 mM EGTA, 1 mM DTT step following extensive washes in 0.25 M KCl in the same buffer with and without ATP.

Measurement of Actin-activated MgATPase Activity—Actin-activated MgATPase activities of the recombinant V HMM and S1 were measured using the method of Pollard and Korn (24) in a buffer containing 10 mM MOPS (pH 7.0), 2 mM $MgCl_2$, 1 mM ATP, 0.1 mM EGTA, and 80 mM KCl unless otherwise specified. The experiments were performed at 25 and 37 °C, and the final myosin concentration in the assay was usually 10–30 nM. The kinetic constants, V_{max} and K_{ATPase} (the actin concentration required to give half-maximal activation of the MgATPase), were determined by a nonlinear least squares fitting of the data to the Michaelis-Menten equation.

Measurement of the Rate of in Vitro Motility—The rates of *in vitro* motility of actin filaments sliding over the expressed myosin fragments were measured as described previously in a buffer consisting of 80 mM KCl, 20 mM MOPS (pH 7.4), 5 mM $MgCl_2$, 1 mM ATP, 0.1 mM EGTA, 50 mM DTT, 2.5 mg/ml glucose, 2 μg/ml catalase, 0.1 mg/ml glucose oxidase at 30 °C (25) unless otherwise specified. The myosin was applied to the flow chamber at concentrations ranging from 0.2 mg/ml to 40 ng/ml. Both uncoated glass and nitrocellulose-coated coverslips were used. The rhodamine phalloidin-labeled (Molecular Probes) actin filaments were imaged with a microchannel plate intensified CCD (ICCD-350F, Videoscope) and recorded on sVHS videotape (Panasonic). The rate of actin filament sliding was quantified using the Cell Trak system (Motion Analysis) as described previously (25).

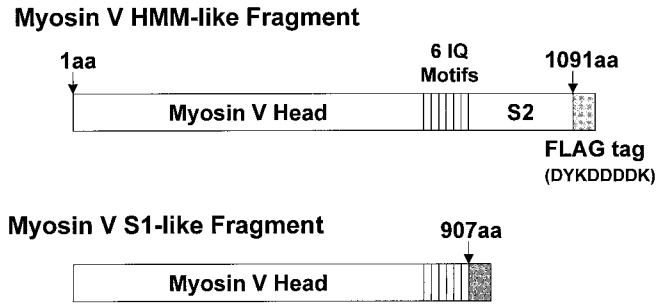

FIG. 1. **Schematic diagrams of expressed myosin V heavy chain fragments.** Stick diagrams depict the domain structure of the expressed myosin V HMM and S1 fragments. The diagram is not drawn to scale. *aa*, amino acid.

Stopped Flow Experiments—ADP release experiments were carried out using SF-2001 Stopped Flow apparatus (KinTek Corp.) in a buffer containing 100 mM KCl, 10 mM MOPS (pH 7.0), 2 mM $MgCl_2$, 0.1 mM EGTA, 1 μM calmodulin at 20 °C. Pyrene-labeled actin was prepared according to the method of Cooper *et al.* (26). One syringe contained 0.2 μM pyrene actomyosin V S1 mixed with various concentrations of ADP, and the other contained 400 μM ATP.

Purification of ATP—It was necessary to purify ATP used in the stopped flow experiments to remove residual ADP contamination. A 10-ml solution of 15 mM ATP (Sigma) in 10 mM triethylamine (pH 7.5) was applied to a 1 × 20-cm column of Q-Sepharose Fast Flow (Amersham Pharmacia Biotech) equilibrated in 10 mM triethylamine (pH 7.5) at 4 °C. The column was developed with a linear gradient (total volume, 400 ml) from 10 to 600 mM triethylamine (pH 7.5). The absorbance at 260 nm was monitored. Fractions were pooled from the peak and descending limb of the curve and rotary evaporated (keeping the temperature less than 15 °C) to dryness. Three washes and evaporations with methanol were carried out at 4 °C to remove residual triethylamine. The ATP powder containing less than 0.05% ADP was dissolved in 10 mM HEPES, pH 7.5 and stored at −80 °C until used.

Electron Microscopy—Samples at approximately 0.1 mg protein/ml were diluted with two parts of glycerol and sprayed on freshly cleaved mica, as described by Uber and Branton (27). The samples were rotary shadowed in a Balzers 301 freeze-fracture unit at an angle of 7° for the platinum. The shadowing was carried out a room temperature.

RESULTS

Purification of the Expressed Myosin V Fragments—Two 3′-truncations of the cDNA encoding the heavy chain of mouse myosin Va were produced (Fig. 1). The first was truncated after codon 1091 to produce an HMM-like molecule containing the motor domain, six IQ motifs, and 184 amino acids of the coiled-coil to form a dimer. The second construct was truncated after codon 907 to produce a monomeric S1-like molecule containing the motor domain and all six IQ motifs that comprise the neck region. In addition, each construct was tagged at its 3′-end with the FLAG epitope to facilitate purification. The heavy chain constructs were cloned into pVL1392 vectors for baculovirus expression and were coinfected into Sf9 cells along with viruses that encode calmodulin and essential light chain. Both proteins were purified from Sf9 cell extracts using affinity chromatography over a FLAG antibody column. The purified proteins are shown in Fig. 2.

The myosin V HMM preparation was rotary shadowed and examined by electron microscopy. The sample showed a mixture of double-headed and single-headed fragments (Fig. 3*A*).

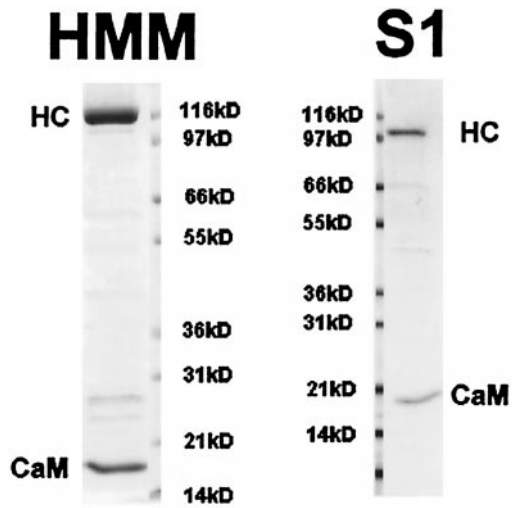

FIG. 2. **SDS-polyacrylamide gel of expressed myosin V fragments.** The purified myosin V fragments were electrophoresed on 15% SDS-polyacrylamide gels. *HC*, heavy chain; *CaM*, calmodulin.

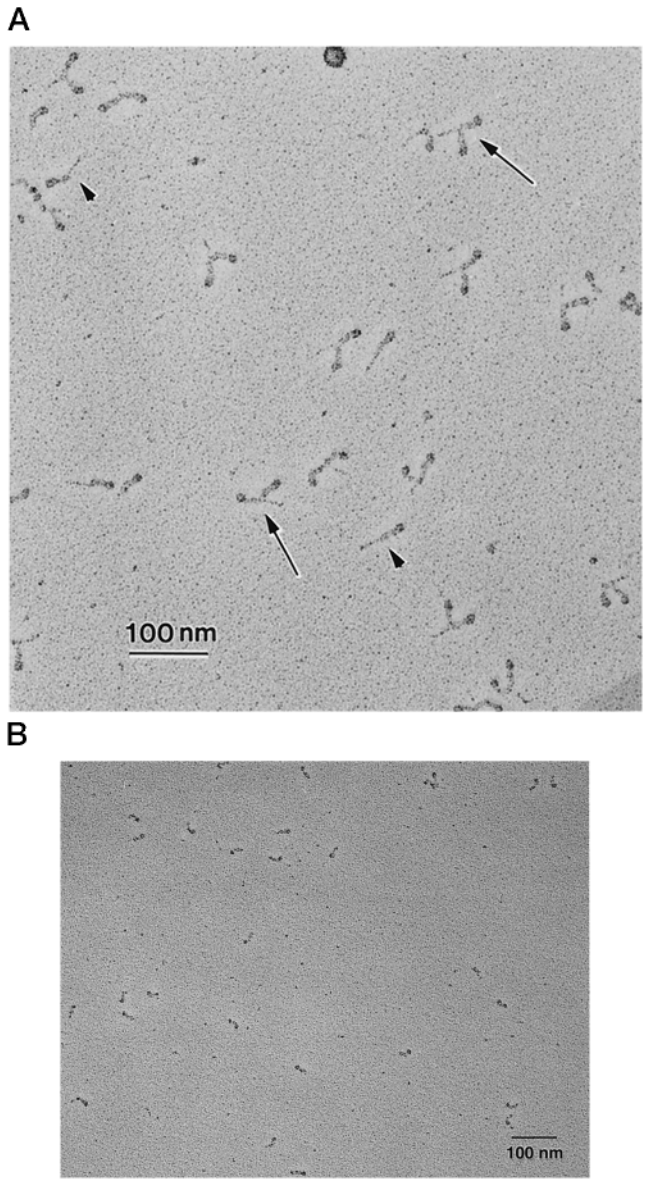

FIG. 3. **Electron microscopy of expressed myosin V fragments.** *A*, HMM was rotary shadowed and viewed by electron microscopy. *Large arrows* show double-headed HMMs, and *small arrows* show single-headed HMMs. Note the presence of a rod in the single-headed preparation. *B*, S1 was rotary shadowed and viewed by electron microscopy.

The single-headed fragments still contained a rod, which suggests that there was proteolysis at the head-neck junction of only one of the two heavy chains leaving the coiled coil region intact. Bands corresponding to the proteolytically produced rods can be seen in Fig. 2 migrating between the 21- and 31-kDa markers. These bands, in addition to the heavy chain band seen at 116 kDa, reacted with the FLAG antibody, indicating that they contained the FLAG epitope at the carboxyl terminus. It is likely that the proteolysis was occurring within the Sf9 cells, because the proteolytic products could be detected in Sf9 cells that were directly lysed into boiling SDS (data not shown). The amount of cleavage was not affected by homogenization in an mixture containing numerous protease inhibitors and did not appear to increase with time. The site of proteolysis is predicted to be within the IQ regions based on the molecular mass of the fragments. The proportion of double-headed HMM varied from preparation to preparation and ranged from 50 to 80%. Not all preparations were checked by electron microscopy, but the amount of degradation on SDS gels was monitored. In contrast, the myosin V S1 preparation appeared homogeneous by rotary shadowing (Fig. 3*B*). Because of this complication, we have chosen to concentrate primarily on the characterization of myosin V S1 in this study but will present some supporting data from the HMM preparation.

A strong band corresponding to calmodulin could be seen with Coomassie Blue staining of 15% SDS-polyacrylamide gels (Fig. 2), and the presence of a very small amount of essential light chain could be detected by Western blotting with an essential light chain antibody in some but not all preparations. Quantitative Western blotting was used to estimate the stoichiometry of essential light chain bound. Western blots were performed on equal amounts of purified recombinant myosin V S1 and purified recombinant nonmuscle myosin IIA HMM using the assumption that the myosin IIA HMM heavy chain binds 1 mol of essential light chain/mol. Comparison of the intensities of the detected bands revealed that myosin V S1 bound much less than 0.1 mol of essential light chain/mol of heavy chain, and, thus, most of the IQ motifs are occupied by calmodulin. In most preparations no essential light chains were detected. Western blots of the cell extract indicated that sufficient essential light chain was being produced (data not shown). Therefore, the substoichiometric binding of essential light chain was not a consequence of limiting levels of essential light chain in the Sf9 cells. This is in contrast to chicken brain myosin V, which copurifies with 1–2 mol of essential light chain/mol. To reconcile the difference between our recombinant mouse proteins and the tissue-purified chicken brain preparation, we purified myosin V from mouse brain. This preparation showed bands corresponding to the heavy chain, calmodulin, and the dynein light chain, but no essential light chain (Fig. 4, *lane 2*). Western blotting of the preparation with an essential light chain antibody also failed to detect any essential light chain in the preparation (Fig. 4, *lane 4*), although it detected the essential light chain of a human platelet actomyosin run on the same gel (Fig. 4, *lane 3*). There is a small amount of a contaminating protein at 55 kDa that probably corresponds to tubulin (23).

MgATPase Activity of Myosin V HMM and S1—Both myosin V HMM and S1 exhibited a low MgATPase activity in the absence of actin of less than 0.04 s^{-1}. Actin markedly activated the MgATPase activity of both fragments (Table I) in a temperature- and ionic strength-dependent manner. At 37 °C the V_{max} values of HMM and S1 were greater than 10 s^{-1}. The

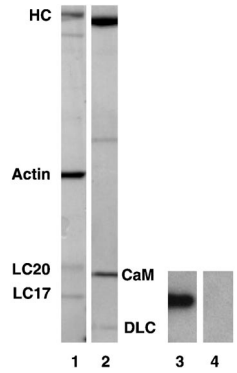

FIG. 4. **Mouse brain myosin V does not contain essential light chain.** *Lanes 1* and *2*, 4–20% SDS-polyacrylamide gel; *lanes 3* and *4*, Western blot with anti-essential light chain antibody. *Lanes 1* and *3*, human platelet actomyosin; *lanes 2* and *4*, tissue-purified mouse brain myosin V. *HC*, heavy chain; *LC20*, regulatory light chain 20 kDa;, essential light chain 17 kDa; *CaM*, calmodulin; *DLC*, dynein light chain.

TABLE I
Actin-activated MgATPase of Expressed Mouse Myosin V Fragments

Expressed myosin V	Temperature	[KCl]	Calmodulin	V_{max}	K_{ATPase}
	°C	mM		s^{-1}	μM
HMM	25	80	ND[a]	3.8 ± 0.6	0.15 ± 0.06
	37	80	ND	11.2 ± 1.1	1.4 ± 0.5
	25	250	ND	1.6 ± 0.3	1.7 ± 0.1
	37	250	ND	1.9 ± 0.1	4.0 ± 1.0
S1	37	80	−	4.0 ± 2.3	2.5 ± 0.4
	25	80	+	4.5 ± 0.4	4.4 ± 1.0
	37	80	+	12.0 ± 3.5	4.0 ± 1.0
	25	250	+	2.0 ± 0.3	24.0 ± 4.3
	37	250	+	6.6 ± 1.1	30.0 ± 4.5

[a] ND, no effect was detected.

K_{ATPase} was remarkably low with a value of 0.15 μM being recorded for the HMM preparation at 25 °C with 80 mM KCl added (total ionic strength, 92 mM). Addition of exogenous calmodulin did not affect the actin-activated MgATPase activity of HMM but did increase that of some preparations of S1. The dependence of this increase in actin-activated MgATPase on calmodulin concentration was measured, and a half-maximal value of about 1 μM was determined (Fig. 5). This probably represents calmodulin binding to one or more of the IQ domains.

Calcium did not activate the MgATPase activity of either myosin V HMM or S1. However, the tissue-purified mouse brain myosin V behaved very differently. In the absence of calcium, its MgATPase activity was 0.1 s^{-1} at 4 μM actin, 80 mM KCl, 37 °C even in the presence of exogenously added calmodulin. The activity was stimulated to 2.2 s^{-1} by the addition of calcium. In the presence of both calcium and exogenous calmodulin, the V_{max} of the tissue-purified mouse brain myosin V was 11 s^{-1}, and the K_{ATPase} was 0.8 μM, similar to values obtained for the recombinant HMM under the same ionic conditions.

Another interesting feature of MgATPase activities is the ionic strength dependence. The K_{ATPase} values for the expressed S1, HMM, and the tissue-purified mouse brain myosin V are remarkably low at 80 mM KCl, and that of HMM is still very low even at 250 mM KCl (Table I).

In Vitro Motility of Myosin V HMM and S1—Myosin V HMM and S1 both supported movement of actin filaments when bound either to a nitrocellulose-coated surface or directly to glass coverslips. The rate of movement was faster for HMM than for S1 under comparable ionic conditions. At 40 mM KCl the rate for HMM was 0.35 ± 0.05 μm/s compared with 0.20 ± 0.03 μm/s for S1. In agreement with previous reports with intact chicken brain myosin V (3), we found that the mouse myosin V fragments could support actin filament sliding even at very low concentrations of myosin V added to the coverslip surface. If flow cells were coated with myosin V fragment concentrations from 20 to 200 μg/ml, actin filaments were rapidly sheared into small fragments. At applied concentrations of 1–2 μg/ml or less, however, actin filaments moved over the surface in a constant manner without shearing. As the surface density decreased, actin filaments translocated through apparently fixed points of attachment to the surface that allowed for Brownian driven rotation of the moving actin filaments. Myosin V HMM supported actin filament sliding at applied concentrations of 40 ng/ml in the presence of methylcellulose, a viscous reagent which suppresses lateral Brownian diffusion. No movement of actin filaments was seen over such sparsely coated surfaces in the absence of methylcellulose. The myosin V S1 is roughly comparable with the myosin V HMM in its ability to support actin filament sliding at low concentrations. The mouse brain myosin V translocated actin filaments somewhat faster than did either the HMM or S1 fragment. The rate of actin translocation was 0.46 μm/s (average of two preparations) for the tissue-purified mouse brain myosin V as compared with 0.27 μm/s for myosin V HMM under comparable conditions (20 mM KCl in the standard motility assay buffer)

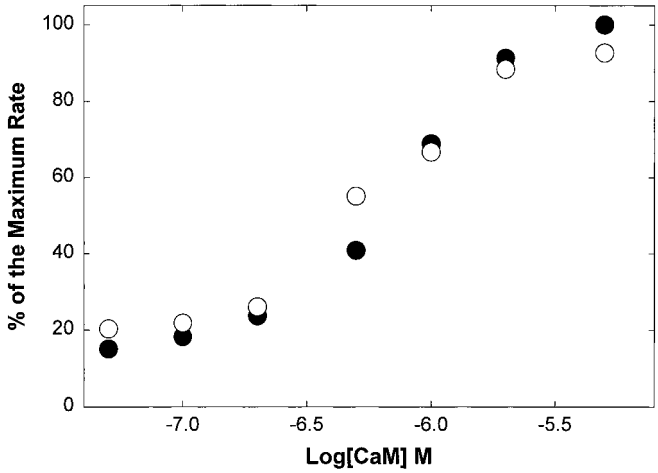

FIG. 5. **Calmodulin dependence of the actin-activated MgATPase activity of myosin V S1.** The MgATPase activity of myosin V S1 was measured in the presence of various concentrations of calmodulin. The symbols represent the data from two independent experiments were the maximal rates were 5 s^{-1} (*closed circles*) and 4 s^{-1} (*open circles*). The conditions of the assay was 20 μM actin, 80 mM KCl, 10 mM MOPS (pH 7.0), 2 mM $MgCl_2$, 0.1 mM EGTA, 1 mM ATP, 37 °C.

The translocation of actin filaments by myosin V fragments was not affected by the presence of tropomyosin. The movement became slower and more erratic as a function of time in calcium containing buffers probably as a result of calmodulin dissociation from the neck of myosin V.

In addition, we have examined the ionic strength dependence of *in vitro* motility. S1 can support the movement of actin filaments even at 400 mM KCl in the absence of methylcellulose (Fig. 6). HMM behaved similarly (data not shown). Thus, the motile properties of myosin V fragments differs dramatically from those of conventional myosin II molecules both in dependence of motility on the myosin concentration and in the ionic strength dependence.

Effect of ADP on in Vitro Motility and MgATPase Activity—The rate of actin filament sliding by myosin V S1 was examined as a function of ADP concentration at a constant ATP concentration of 200 μM (Fig. 7). As a comparison, the same experiment was conducted with rabbit skeletal muscle HMM. ADP

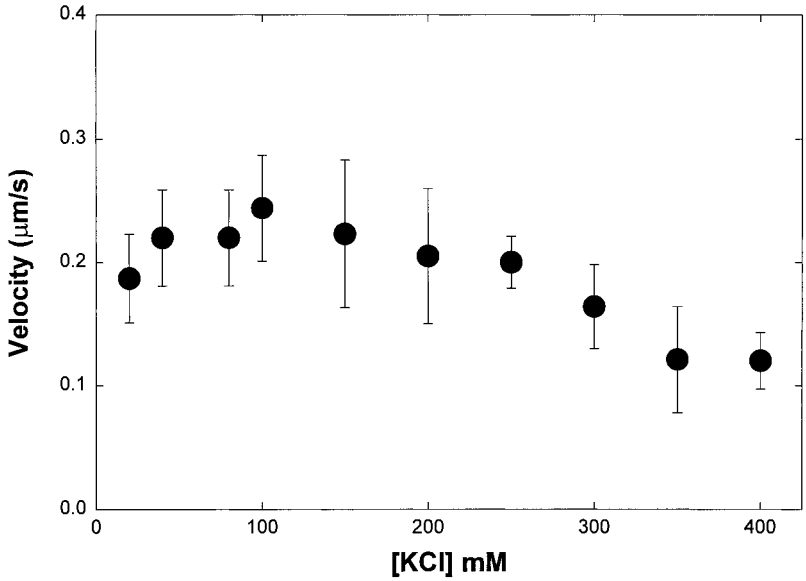

FIG. 6. **Ionic strength dependence of actin filament sliding.** The *in vitro* motility of actin filaments moving over a surface of S1 was measured as a function of added KCl. Actin filaments moved constantly at all ionic strengths tested. The concentration of S1 applied to the surface varied from 2 μg/ml at the lowest ionic strengths to 200 μg/ml at the highest ionic strengths.

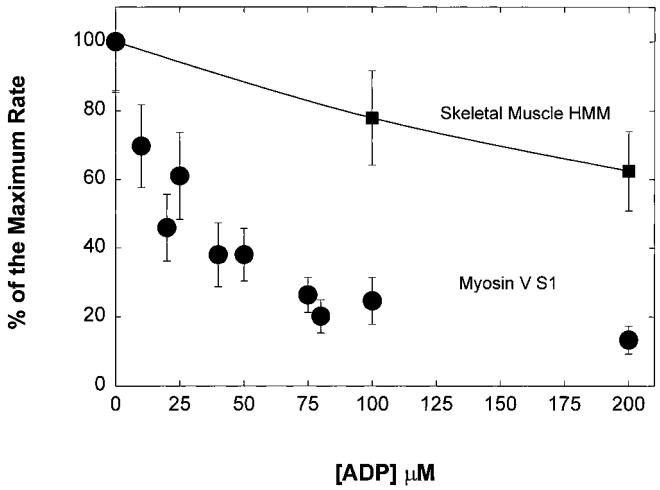

FIG. 7. **ADP inhibition of *in vitro* motility.** The effect of ADP on the rate of *in vitro* motility of myosin V S1 was determined by varying the ADP concentration. Note that the ATP concentration for this experiment was 200 μM. Actin filaments moved constantly at all ADP concentrations. The maximal rate of actin filament sliding induced by myosin V S1 was 0.12 μm/s in this experiment, and that of skeletal muscle HMM was 3 μm/s. For comparison the effect of ADP on the translocation of actin filaments by rabbit skeletal muscle HMM is shown. The conditions for this experiment was 40 mM KCl, 20 mM MOPS (pH 7.4), 5 mM $MgCl_2$, 0.1 mM EGTA, 0.2 mM ATP, 50 mM dithiothreitol, 2.5 mg/ml glucose, 2 μg/ml catalase, 0.1 mg/ml glucose oxidase at 30 °C.

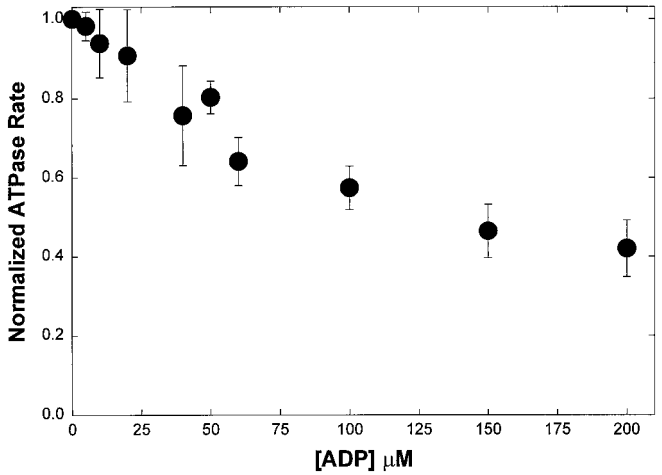

FIG. 8. **ADP inhibition of the actin-activated MgATPase activity of myosin V S1.** The effect of ADP on the actin-activated MgATPase activity of myosin V S1 was determined by addition of various concentrations of ADP. The maximal ATPase rate in this experiment was 6 s^{-1}. The ATP concentration in this experiment was 200 μM. The conditions of the assay were 80 mM KCl, 10 mM MOPS (pH 7.4), 2 mM $MgCl_2$, 0.1 mM EGTA, and 0.2 mM ATP at 37 °C.

inhibited the rate of actin filament sliding of the myosin V S1 much more potently than it did that of rabbit skeletal muscle HMM. The rate of actin filament sliding was reduced to 50% of its maximal value at an ADP concentration of about 25 μM for myosin V. By contrast, greater than 200 μM ADP was needed to inhibit the rate of actin filament sliding by skeletal muscle HMM only by a comparable amount. Myosin V HMM behaved similarly to myosin V S1 in this assay.

ADP also inhibited the actin-activated MgATPase activity of myosin V S1 (Fig. 8). Over the range of ADP concentrations studied, no clear plateau in activity was determined, but it is clear that the ATPase activity was inhibited over a similar range of ADP concentrations as was used to inhibit the *in vitro* motility.

Rate of ADP Release from Actomyosin V S1 and Affinity of Actomyosin V S1 for ADP—Stopped flow spectrofluorimetry was used to measure the rate of ADP release from an actomyosin S1 complex. When pyrene-labeled actin binds to myosin, the fluorescent signal is quenched by 80% (28). The complex of actin and myosin V S1 dissociated at a rate of 46 s^{-1} when rapidly mixed with 200 μM ATP (Fig. 9*A*). At ADP concentrations between 0.05 and 5 μM, the ATP-induced dissociation of ternary complexes of actin, myosin V S1, and ADP occurred as a bi-exponential process (Fig. 9*A*). The fast phase had a rate constant similar to that for the dissociation of acto-S1 by ATP and corresponded to dissociation of the acto-S1 complex that had no ADP bound. The amplitude of the slow phase increased with increasing ADP concentrations and occurred at a rate of 11.5 ± 0.8 s^{-1}. The percentage of slow phase amplitude was plotted *versus* the ADP concentration (Fig. 9*B*). The data were fitted by a hyperbola that yielded a K_d of 0.77 μM for the binding of ADP to pyrene-actomyosin V S1. It was necessary to use ATP that has been column purified for this experiment because commercial sources of ATP contained 0.7–1.3% ADP as measured by a linked assay using pyruvate kinase, phosphoenolpyruvate, NADH, and lactic-acid dehydrogenase. The kinetic experiments were performed at 20 °C to facilitate com-

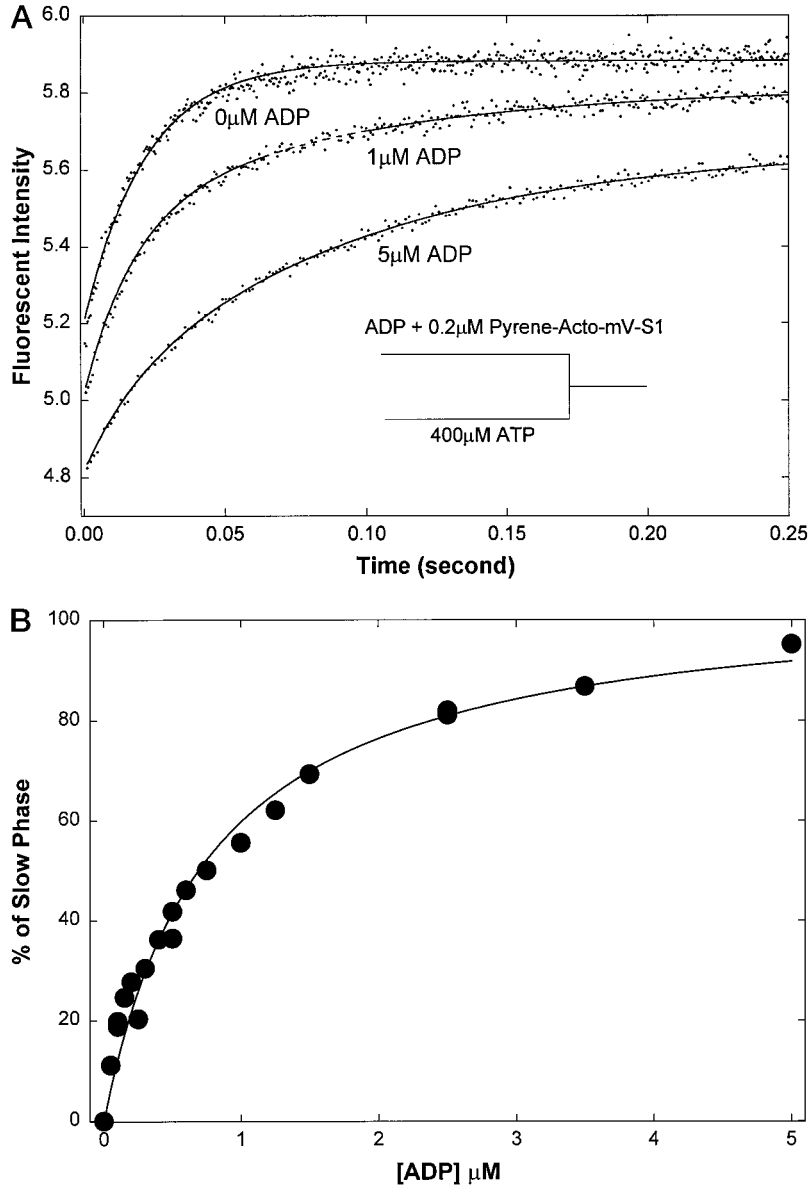

FIG. 9. **ADP inhibition of ATP-induced dissociation of pyrene-acto-S1 complex.** *A*, observed fluorescence changes upon mixing of 200 μM ATP with 0.1 μM pyrene-acto-S1 and 0, 0.5, and 2.5 μM ADP. The *curve* in the absence of ATP was fit to a single exponential with a rate of 45.8 s^{-1}. The *curves* in the presence of ADP were fit to the sum of two exponentials. For the experiment in which 0.5 μM ADP was used, the amplitudes and rates were -0.451 mV, 56 s^{-1}, and -0.351 mV, 11.6 s^{-1}. For the experiment in which 2.5 μM ADP was used, the amplitudes and rates were -0.162 mV, 56.4 s^{-1} and -0.682 mV, 10.8 s^{-1}, respectively. The experiment was determine at ADP concentrations ranging from 0.05 to 5 μM. *B*, [ADP] dependence of the slow phase amplitude percentage. The best fit to a hyperbola gave a K_d values of 0.77 μM. Control experiments (not shown) demonstrated that ADP did not cause dissociation of the pyrene-acto-S1 complex. The conditions of the reaction are given under "Experimental Procedures." Note that the ATP and ADP concentrations mentioned here are the concentrations after mixing.

parison with numerous published studies of other myosins. The V_{max} and K_{ATPase} of the steady state MgATPase activity under these conditions were 3.3 s^{-1} and 4.3 μM, respectively. Thus, the rate constant for release of ADP is quite close to the steady state rate, and ADP release may be partially rate-limiting.

DISCUSSION

Studies of single-headed and double-headed myosin fragments have been essential to elucidation of myosin function. Here, we report the characterization of such fragments from an unconventional myosin, mouse myosin Va. There are several advantages for using recombinant expression of myosin fragments *versus* tissue purification of the myosin. We can purify up to 7 mg of myosin fragment from a 1-liter culture of Sf9 cells using FLAG affinity chromatography, whereas tissue purification of chicken brain myosin V yields less than 1 mg/100 g of brain (23). Furthermore, the ability to study the kinetics of a single-headed fragment greatly simplifies the initial kinetic characterizations, particularly of a myosin that might move processively on an actin filament, and thus involves coordinated action of the two heads.

Chicken brain myosin V has previously been purified and shown to contain calmodulin and essential light chain as subunits in addition to a lower molecular mass subunit that is identical to a previously reported dynein light chain (3, 5). The expressed recombinant mouse myosin V fragments did not bind essential light chain, even though it was coexpressed along with calmodulin and heavy chain in the Sf9 cells. We, therefore, purified mouse brain myosin V to examine its subunit composition. It contains myosin heavy chain, calmodulin, and dynein light chain but no essential light chain. Thus, the composition of the neck regions of myosin V from these two species must be different.

Myosin V has three unique kinetic features compared with myosin II. First, its apparent affinity for actin in the presence of ATP is much higher than that of most myosin II subfragments. Second, its actin-activated MgATPase activity is much less sensitive to increasing ionic strength than that of rabbit skeletal muscle subfragments. The K_{ATPase} of the V HMM is 1 μM or less at 80 mM KCl concentration (ionic strength, 92 mM) and is still remarkably low even at the unphysiologically high KCl concentration of 250 mM (ionic strength, 262 mM). The high affinity of myosin V for actin is related to its ability to translocate actin at low myosin surface densities *in vitro*, another feature that distinguishes myosin V from myosin II class mol-

ecules. Third, myosin V has a very high affinity of ADP.

The V_{max} of the actin-activated MgATPase activity of mouse myosin V HMM and S1 is quite high (10–12 s^{-1}) and was not activated by calcium. In fact, calcium exerted a slight inhibitory effect in the absence of calmodulin. In contrast, the actin-activated MgATPase activity of tissue-purified mouse brain myosin V is very low in the absence of calcium (0.1 s^{-1} at 4 μM actin, 80 mM KCl, 37 °C) but is activated to a V_{max} of 11 s^{-1} with a K_{ATPase} of 0.8 μM under conditions that were the same except for the addition of calcium and exogenous calmodulin. Calmodulin by itself is not sufficient to stimulate the activity. The requirement for calcium is similar to that of chicken brain myosin V. Interestingly, neither tissue-purified myosin V preparations require calcium for *in vitro* motility (4). Nascimento *et al.* (4) digested chicken brain myosin V with calpain that cleaves at two sites. One site is in the tail, which produces an HMM like fragment, whereas the other site is probably at a flexible loop in the head. This fragment, similar to our recombinant mouse myosin V fragments, has a calcium-independent MgATPase activity. The lack of calcium dependences of the actin-activated MgATPase of myosin V subfragments suggests that the myosin V tail may be able to inhibit the activity when the protein is in solution. This could be an important physiological regulation of the molecule, which requires further study.

The slow rate of ADP release and the apparent high affinity of myosin V for ADP in the presence of actin is of considerable interest. The ADP release rate for most myosins is considerably faster than the V_{max} of the steady state MgATPase rate in the presence of actin (see Ref. 1 for review). It has been proposed that the rate of release of ADP limits the rate of unloaded actin filament sliding, whereas some other kinetic step such as phosphate release or ATP hydrolysis controls the MgATPase rate (29). The ADP release rate for mouse myosin V fragments at 20 °C is about 11 s^{-1}, which is only about three times greater than the V_{max} of the steady state MgATPase rate at this temperature, and, thus, ADP release may be partially rate-limiting. Indeed, we find that ADP inhibits both the rate of actin filament sliding and the actin-activated MgATPase activity over the same range of concentrations. The K_d of 0.77 μM determined for binding of ADP to the pyrene-acto-S1 complex is the highest ADP affinity determined for a myosin to date. By comparison, ADP binds to smooth muscle pyrene-acto-S1 and to pyrene-acto-rat myosin I (myr1) with K_d values of 5 μM and $\leq$10 μM respectively (30, 31). In contrast to these relatively low values, ADP binds to pyrene-acto-S1 from rabbit skeletal muscle myosin with a K_d of 200 μM (32). The midpoint of the inhibition of the MgATPase activity and of the *in vitro* motility occurs at 25–50 μM ADP, which is close to the physiological concentration, and it may be that ADP modulates the activity of myosin V *in vivo*.

Myosin V functions as a vesicle motor *in vivo* (14–16, 18, 33). From this study and others it is clear that its kinetics are attuned to this type of function (3, 4, 20). Only a few myosin V molecules are required to move actin filaments in the *in vitro* motility assays. Indeed, Mehta *et al.* (20) recently proposed that a single myosin V molecule is able to take several steps while attached to actin, in a manner already described for the microtubule-dependent motor, kinesin (34, 35). The ability of a myosin to move processively on actin filaments is a function of several kinetic phenomena, the high affinity for actin, and the slow release of ADP compared with the steady state MgATPase rate. Together, this type of kinetics assure that the myosin has a high duty cycle ratio, *i.e.* is attached for a significant fraction of its kinetic cycle. Thus, a two-headed myosin V molecule might be able to remain bound to actin via one head while the other head is detached and repriming for its next power stroke. Even if myosin V does not have the same ability to undergo long range processive movements as has been demonstrated with fluorescently labeled kinesin molecules on microtubules (35), its kinetics are such that only a few myosin V molecules at the interface between a vesicle and an actin filament should be sufficient to drive transport.

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