Calmodulin Dissociation Regulates Brush Border Myosin I (110-kD–Calmodulin) Mechanochemical Activity In Vitro

Kathleen Collins,* James R. Sellers,‡ and Paul Matsudaira*

*Department of Biology, Massachusetts Institute of Technology, and Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142; and ‡Laboratory of Molecular Cardiology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

Abstract. 110-kD–calmodulin, when immobilized on nitrocellulose-coated coverslips, translocates actin filaments at a maximal rate of 0.07–0.1 μm/s at 37°C. Actin activates MgATPase activity >40-fold, with a K_m of 40 μM and V_{max} of 0.86 s^{-1} (323 nmol/min/mg). The rate of motility mediated by 110-kD–calmodulin is dependent on temperature and concentration of ATP, but independent of time, actin filament length, amount of enzyme, or ionic strength. Tropomyosin inhibits actin binding by 110-kD–calmodulin in MgATP and inhibits motility. Micromolar calcium slightly increases the rate of motility and increases the actin-activated Mg-ATP hydrolysis of the intact complex. In 0.1 mM or higher calcium, motility ceases and actin-dependent MgATPase activity remains at a low rate not activated by increasing actin concentration. Correlated with these inhibitions of activity, a subset of calmodulin is dissociated from the complex. To determine if calmodulin loss is the cause of calcium inhibition, we assayed the ability of calmodulin to rescue the calcium-inactivated enzyme. Readdition of calmodulin to the nitrocellulose-bound, calcium-inactivated enzyme completely restores motility. Addition of calmodulin also restores actin activation to MgATPase activity in high calcium, but does not affect the activity of the enzyme in EGTA. These results demonstrate that in vitro 110-kD–calmodulin functions as a calcium-sensitive mechanoenzyme, a vertebrate myosin I. The properties of this enzyme suggest that despite unique structure and regulation, myosins I and II share a molecular mechanism of motility.

I N the brush border of intestinal epithelium, a complex of 110-kD protein and calmodulin (110-kD–CaM)[1] laterally bridges the microvillar core to the cell membrane in a connection disrupted by ATP (Matsudaira and Burgess, 1979). Biochemical and structural studies have provided strong evidence that this complex is myosin-like (for reviews of myosin, see Warrick and Spudich, 1987; Korn and Hammer, 1988). In solution, the complex was shown to have Mg-ATPase activity weakly stimulated (two- to threefold) by actin as well as actin-independent K$^+$-EDTA ATPase and CaATPase activities (Collins and Borysenko, 1984; Conzelman and Mooseker, 1987; Krizek et al., 1987; Swanljung-Collins et al., 1987). By EM, 110-kD–CaM decorates single actin filaments (Coluccio and Bretscher, 1987) and actin bundles in vivo (Matsudaira and Burgess, 1982) and in vitro (Coluccio and Bretscher, 1989) with an arrowhead polarity reminiscent of the myosin subfragment-1 (S1). After rotary shadowing, both 110-kD–CaM and S1 appear as globular proteins of similar dimensions (Conzelman and Mooseker, 1987).

Homology in the primary sequence of 110 kD and myosin has also been demonstrated. mAbs that recognize epitopes of the ATP- and actin-binding domains of skeletal muscle myosin also recognize 110 kD (Carboni et al., 1988). On the nucleotide level, a cDNA clone of the bovine 110-kD homologue shows extensive homology with myosin S1 (Hoshimaru and Nakanishi, 1987). The sequence of the bovine 110-kD gene suggests that the COOH-terminal region of the protein lacks potential to form an α-helical coiled coil. Because this region mediates heavy chain multimerization in myosin, its absence in the 110-kD protein suggests that 110-kD subunits can not polymerize into myosin-like filaments. In the size and sequence of its heavy chain, 110-kD–CaM most closely resembles *Acanthamoeba* (Pollard and Korn, 1973) and *Dictyostelium* (Cote et al., 1985) myosin I. Myosin I in its single-headed structure contrasts with conventional, double-headed myosin II (for comparison of myosin I and II, see Korn and Hammer, 1988).

Despite their similarity in primary structure, 110-kD–CaM and *Acanthamoeba* myosin I differ in several important properties. Actin binds 110-kD–CaM at an ATP-dependent actin-binding site (Coluccio and Bretscher, 1988; Carboni et al., 1988), but binds *Acanthamoeba* myosins IA and IB at both ATP-dependent and independent binding sites (Korn and Hammer, 1988). Furthermore, the 110-kD heavy chain

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1. *Abbreviations used in this paper:* 110-kD–CaM, the complex of 110-kD protein and calmodulin; pCa, −log of free calcium concentration; S1, myosin subfragment-1.

is associated with two to four calmodulin subunits rather than a single light chain, as in *Acanthamoeba* myosin I, or two specialized light chains, as in myosin II. The role of the calmodulin subunits in the regulation of the structure and function of the 110-kD protein is not known, nor is the exact stoichiometry of the 110-kD–CaM complex.

An intriguing feature of myosin I, and of 110-kD–CaM, is the association of the protein with membranes. Whereas nonmuscle myosins are cytosolic, localized to actin networks within the cell, myosin I in *Acanthamoeba* is localized by antibodies to the plasma membrane (Gadasi and Korn, 1980). In motile *Dictyostelium* cells, myosin I localizes to the cell's leading edge while myosin II localizes more diffusely in the rear of the cell (Fukui et al., 1989). *Acanthamoeba* myosin I has also been shown to cofractionate with and move vesicles in vitro (Adams and Pollard, 1986), and both myosins IA and IB have been shown to bind NaOH-stripped *Acanthamoeba* vesicles and vesicles of pure phospholipid (Adams and Pollard, 1989). The 110-kD protein also localizes to plasma membranes, in particular to the apical, brush border membrane, but details of the localization differ between reports.

Although it is clear that the 110-kD–CaM complex serves as a membrane linkage in intestinal epithelium, its myosin-like enzymatic properties suggest that the linkage could be part of a motile system. Recently Mooseker and Coleman (1989) have described a very slow rate of movement of polymer beads coated with 110-kD–CaM on actin cables in the *Nitella*-based in vitro motility assay. Also, Mooseker et al. (1989) have measured a slightly higher rate of movement of membrane preparations containing 110-kD–CaM in the same assay. We have purified the 110-kD–CaM complex and correlated its motility with ATPase activity and structure. In our preparation of the complex, the MgATPase activity increases over 40-fold in the presence of actin. Using the sliding filament in vitro motility assay, 110-kD–CaM (brush border myosin I) translocates actin with a maximal rate of 0.07–0.1 μm/s at 37°C, a rate similar to that found for other cytoplasmic myosins. Tropomyosin inhibits motility and inhibits the association of brush border myosin I with actin filaments, opposite its activating effect on smooth and nonmuscle myosin II. Micromolar calcium modestly stimulates motility and MgATPase activity; surprisingly, 0.1 mM calcium halts motility and eliminates actin activation of Mg-ATPase activity. The inhibition of myosin I activities in calcium is correlated with the loss of a subset of calmodulin from the complex, and both activities are restored with calmodulin readdition.

Materials and Methods

Purification of Proteins

Myosin I was purified from the intestines of 12 freshly slaughtered chickens after a protocol extensively modified from Swanljung-Collins et al. (1987). All steps except release of epithelial cells and FPLC were done at 4°C or with chilled buffers, and protease inhibitors (~0.1 mM PMSF, 1 KIU/ml aprotinin, 0.2 μg/ml leupeptin) were used in brush border homogenization, wash, and extraction steps. Intestines were slit open, washed in 10 mM imidazole, 140 mM NaCl (pH 7.4), and cut into segments. Segments were stirred in PBS (pH 7.3) supplemented with 100 mM sucrose, 10 mM EDTA, and 0.1 mM PMSF to release epithelial cells from the submucosa. After filtration through a colander, cells were collected by low speed centrifuga-

tion and the pellet resuspended in 10 mM imidazole (pH 7.3), 10 mM EDTA, 5 mM EGTA. The cells were homogenized with a Polytron for 30 s at half speed. Brush borders and nuclei were collected by low speed centrifugation and washed in 10 mM imidazole (pH 7.3), 1 mM EGTA, 75 mM NaCl, 5 mM EDTA. The pellet was resuspended in an equal volume of 20 mM imidazole (pH 7.3), 20 mM $MgCl_2$, 2 mM EGTA, 400 mM NaCl, and the myosin I released with three additions of 5 mM ATP over 20 min. The extract was centrifuged in a rotor (model Ti45; Beckman Instruments, Inc., Palo Alto, CA) at 40,000 rpm for 30 min and the supernatant adjusted to 0.6 M NaCl. The high salt supernatant was concentrated by addition of sephadex G75 dry resin, adjusted to 5 mM ATP and 1 mM DTT, and loaded on a Sepharose CL-4B column (2.6 cm × 90 cm) equilibrated and run in 10 mM imidazole (pH 7.3), 1 mM $MgCl_2$, 1 mM EGTA, 0.6 M NaCl, 5% sucrose, 3 mM NaN_3 with 80- and 40-ml rinses of 10 mM imidazole (pH 7.3), 5 mM $MgCl_2$, 1 mM EGTA, 0.6 M NaCl, 1 mM DTT, 5 mM ATP immediately before and after the column load. Fractions containing myosin I were identified by SDS-PAGE in gradient minigels (Matsudaira and Burgess, 1978), dialyzed into CM buffer (10 mM imidazole, pH 7.3, 0.1 mM $MgCl_2$, 1 mM EGTA, 50 mM NaCl, 10% sucrose) containing 0.1 mM PMSF, loaded on a Sepharose CM column (2.6 cm × 16 cm) preequilibrated in CM buffer with 1 mM DTT and 1 mM ATP, and eluted in CM buffer containing 500 mM NaCl. Fractions containing myosin I were identified by SDS-PAGE, pooled, adjusted to 5 mM DTT, filtered, diluted with 1 vol Q buffer (10 mM imidazole, pH 7.3, 0.1 mM $MgCl_2$, 1 mM EGTA, 100 mM NaCl, 10% sucrose), and loaded on a MonoQ HR5/5 FPLC column. Elution with a gradient to 0.5 M NaCl in Q buffer yields up to 2 mg of pure myosin I in <2 d. This quantity represents 10% of the estimated myosin I present in the brush borders, with a 10^5-fold purification. Protein concentration was determined by BCA assay (Pierce Chemical Co., Rockford, IL) using BSA as a standard.

Brush border myosin II was purified from Sepharose CL-4B column fractions of the ATP extract described above. Myosin II-containing fractions were identified by SDS-PAGE, pooled, and dialyzed against 10 mM MOPS, pH 7.0, 25 mM KCl, 10 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT, 3 mM NaN_3 for 20 h. Precipitated myosin was collected by gravity or low speed centrifugation. If necessary, myosin was dissolved in high salt, high ionic strength buffer with MgATP and centrifuged at >100,000 g to remove contaminating actin.

Actin was prepared from acetone powder of rabbit muscle following the procedure of Taylor and Weeds (1976) or Spudich and Watt (1971) as modified by Eisenberg and Kielley (1974). Bovine brain calmodulin was purified following the procedure of Klee (1977) or obtained from Sigma Chemical Co. (St. Louis, MO). Turkey gizzard smooth muscle tropomyosin was purified following the procedure of Umemoto et al. (1989).

Antibodies

Rabbit polyclonal antiserum (R87.5 and R88.5) immunoreactive against the 110-kD protein was made against pure myosin I. Antibodies were purified by affinity chromatography on myosin I conjugated to Sepharose. After washing antibody-bound beads in PBS containing 1 M NaCl, affinity-purified IgG was eluted with 0.2 M glycine, pH 2.7, brought to neutral pH with 1 M Tris, pH 8.5, and dialyzed against PBS containing 3 mM NaN_3. Aliquots were stored at −80°C and thawed before use.

Polyclonal antiserum to brush border myosin II was made against protein purified from turkey intestine by the method of Sellers et al. (1988).

Western Blots

After SDS-PAGE, protein samples (0.2–2.0 μg) were transferred to nitrocellulose membranes for 0.75 A × h in a transphor apparatus (Hoefer Scientific Instruments, San Francisco, CA). The membranes were processed following standard conditions. Primary antisera were used at a concentration of 0.1 μg/ml affinity-purified IgG for anti-110 kD and a 1:500 dilution of serum for antimyosin II, and visualized with alkaline-phosphatase conjugated goat anti-rabbit IgG (ICN K & K Laboratories, Inc., Plainview, NY).

ATPase Assay

Myosin I ATPase activity was measured at 37°C using the Pollard and Korn (1973) modification of the organic extraction protocol of Martin and Doty (1949) with minor changes. [γ-^{32}P]ATP (NEN, Boston, MA) was diluted from a 10-mM stock solution to a final specific activity of 5 μCi/ml for the assays. Quenched samples were counted by liquid scintillation. For each rate measurement, either four (Fig. 5) or eight (Table I) time points were

taken. Hydrolysis rate and error were calculated using the Macintosh Statworks program. The rate of hydrolysis remained linear for at least 40 min, the latest time point measured. Reactions for double-reciprocal plot data were done with >40 μg/ml myosin I in final buffer conditions of standard ionic strength: 10 mM imidazole, pH 7.0 at 37°C, 2 mM $MgCl_2$, 0.1 mM EGTA, 0.4 mM DTT, 1 mM ATP. Reactions for calcium-regulation experiments were done with myosin I at >15 μg/ml (Fig. 5) or 8 μg/ml (Table I) concentration in final buffer conditions of 45 mM ionic strength: 10 mM imidazole, pH 7.2 at 37°C, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM ATP, with $CaCl_2$, KCl, and KOH to the specifications of a program for buffered free calcium kindly provided by Robert E. Godt (University of Georgia). The program's stability constants were taken from Godt and Lindley (1982) and Godt and Nosek (1989). Buffer of −log of free calcium concentration (pCa) 3 was created by addition of 1 mM $CaCl_2$ to the pCa 4 buffer. Myosin I was used for only 2 wk after purification, and rate of ATP hydrolysis per mole myosin was independent of myosin concentration over the range used.

Motility Assay

The motility assay procedure was mainly as in Toyoshima et al. (1987). Flow chambers with volumes of ~20 μl were created between a glass slide and an inverted nitrocellulose-coated coverslip using Apiezon grease and slivers of coverslip as spacers. The flow chamber was first filled with myosin I at concentrations of 0.05->1.0 mg/ml and was allowed to stand for 0.5-2 min. The flow chamber was then washed by addition of 100 μl of buffer M (10 mM imidazole, pH 7.2 at 37°C, 20 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT) containing 1 mg/ml BSA. After 2 min the chamber was washed with buffer M containing 10 nM actin complexed with rhodamine-labeled phalloidin. After 1 min, motility was initiated by addition of buffer M containing 1 mM ATP, 2.5 mg/ml glucose, 0.1 mg/ml glucose oxidase, and 0.02 mg/ml catalase. The last three components were added to reduce photobleaching (Kishino and Yanagida, 1988). Rhodamine-labeled actin was prepared as in Kron and Spudich (1986). Glucose oxidase and catalase were from Sigma Chemical Co.

Slides were placed on a temperature-equilibrated heat stage fixed on a standard microscope equipped with epifluorescence optics and tetramethyl-rhodamine filters (Carl Zeiss, Inc., Thornwood, NY). Filaments were observed with a 100× 1.3 NA oil-immersion objective (Neofluor), mounted KS1380 image intensifier and VS2000N video camera (Videoscope International Ltd., Washington, DC), and were recorded on an AG6300 VHS video recorder (Panasonic). For each assayed motility condition, the movement of filaments was recorded in 3-min intervals for several microscope fields. In each field at least ten randomly selected filaments were quantified for every rate measurement, with rate of movement determined by a program developed by Dr. Steven Block and described in Sheetz et al. (1986). Under all conditions for which a rate was determined, temperature was kept at 37°C unless otherwise indicated, and more than 98% of filaments observed were motile. Myosin I was used for up to 5 wk after purification; we observed no change in rate or regulation of motility over this time.

Glycerol Gradients

Myosin I was centrifuged through 4.4-ml glycerol gradients (15-30%) at 40,000 rpm for 21 h at 4°C in a rotor (model SW50-1; Beckman Instruments, Inc.). Addition of 0.1% NP-40 to myosin samples had no effect on calmodulin association or the extent of sedimentation, so it was routinely included to discourage loss of the 110-kD protein by precipitation. Approximately 200-μl (four-drop) fractions were collected from the bottom of the gradient. After addition of 5× sample buffer, fractions were analyzed by SDS-PAGE on 5-25% polyacrylamide gradient minigels. Glycerol gradient buffers, mixed fresh before each use, were similar to those of motility and ATPase pCa experiments, differing only in the addition of glycerol and 1 mM DTT. The gradients were calibrated using catalase (240 kD), BSA (68 kD), ovalbumin (44 kD), and cytochrome c (14 kD).

Results

MgATPase Activity of Myosin I

The myosin I complex was extracted with MgATP from a brush border–enriched epithelial cell fraction and further purified by gel filtration and ion-exchange chromatography;

inclusion of EGTA in all buffers reduced the dissociation of calmodulin subunits (see Materials and Methods). Myosin I purified by this procedure is typically stable in activity for several weeks, and no significant proteolytic degradation is observed. Immunoblots (Fig. 1) show that purified myosin I was not contaminated with myosin II. Polyclonal antiserum affinity purified to brush border myosin I did not cross react with the 200-kD brush border myosin II, and polyclonal antisera specific for the 200-kD brush border myosin (Fig. 1) or platelet myosin (not shown) did not cross react with myosin I. The myosin II antisera would have detected 1 ng of contaminating protein, an amount <1/250th of the concentration of myosin I. Similarly, we can rule out contamination of our preparation with the S1 fragment of brush border myosin II: the myosin II antiserum recognizes the ~90-kD S1 domain equally as well as the 140-kD rod domain, indicating that myosin II S1 in the myosin I preparation would have been detected in these immunoblots.

The MgATPase activity of the purified enzyme was measured at actin concentrations up to 80 μM (Fig. 2). At 37°C in EGTA, the MgATPase activity in the absence of actin was 0.02 s^{-1}. Actin stimulated the ATPase activity >40-fold in a simple hyperbolic manner: from a double-reciprocal plot of the kinetics, we calculate a K_m of 40 μM and a V_{max} of 0.86 s^{-1} (323 nmol/min/mg) (Fig. 2, inset). The soluble S1 and heavy meromyosin domains of vertebrate cytoplasmic myosin IIs have similar MgATPase activities, catalyzing a low rate of ATP hydrolysis in the absence of actin and displaying simple Michaelis-Menten kinetics of actin activation (Sellers et al., 1988).

Translocation of Actin Filaments on Myosin I-coated Coverslips

The 40-fold actin activation of ATPase activity described above suggested that the homology of the 110-kD protein to myosin included a strong conservation of enzymatic properties. To test whether this conservation included motility, we assayed the ability of enzyme immobilized on nitrocellulose-coated coverslips to translocate rhodamine-labeled phalloidin-stabilized actin filaments. Video monitoring of the fluorescent filaments showed enzyme-dependent attachment of filaments to the coverslip in the absence of ATP and movement of filaments with the addition of ATP. In 30-s time-lapse images recorded on videotape (Fig. 3), the position of filaments moved continuously: some filaments cross paths, diverge, or break apart, and some filaments dissociate from or attach to the coverslip in the period of taping. Under standard conditions at 37°C, the velocity of movement was determined for eight different preparations of myosin I. Rates ranged from 0.07 to 0.1 μm/s, compared with the 0.1 μm/s measured for platelet myosin (S. Umemoto and J. Sellers, personal communication) and the 0.08 μm/s we measure for purified brush border myosin II under the same conditions.

The rate of motility was constant for >2 h after addition of ATP (Fig. 4 a) and for filaments ranging in length from less than 1 μm to greater than 8 μm (Fig. 4 b). The minimum amount of enzyme per coverslip necessary to promote motility was determined by dilution of the myosin I added to the flow cell. At concentrations below 50 μg/ml, with 25 μl of sample loaded per coverslip, actin filaments bound the coverslip less securely. The rates of motility for dilutions to
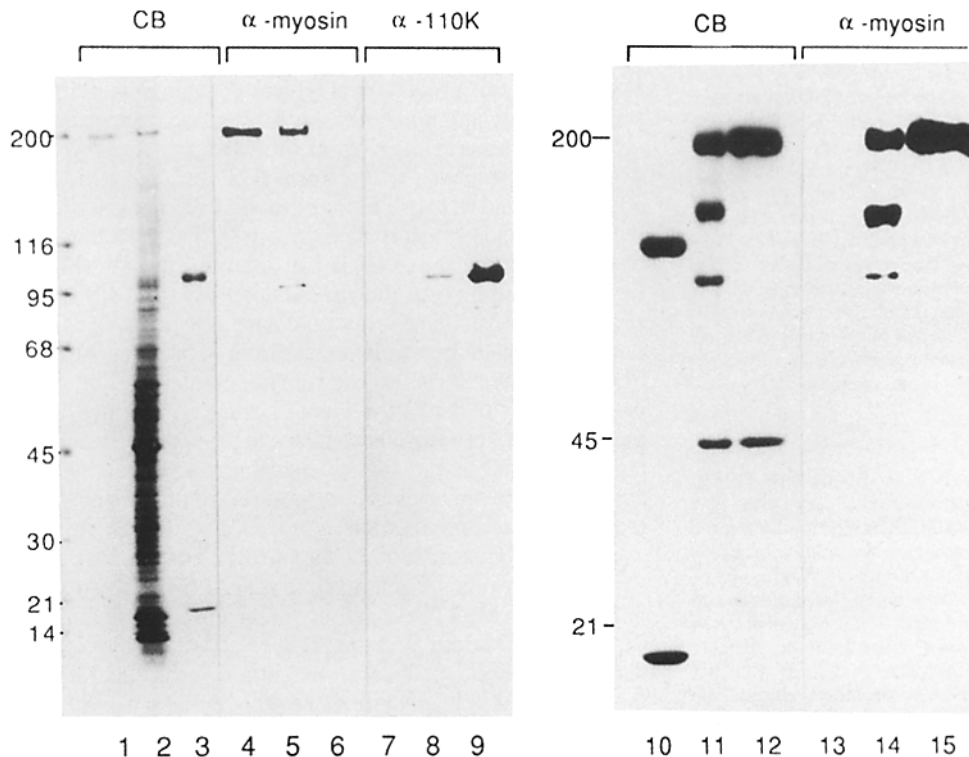
Figure 1. Myosins of brush border. Identically loaded protein samples of brush border myosin II (lanes *1, 4, 7*), total brush border homogenate (lanes *2, 5, 8*), and purified myosin I (lanes *3, 6, 9*) were electrophoresed on a single 5–20% polyacrylamide gradient minigel. One-third of the gel was stained with 0.25% Coomassie blue (lanes *1–3*). Marker molecular weights (in kilodaltons) are indicated on the left. Purified myosin I consists of 110- and 18-kD polypeptides with no detectable degradation of the 110-kD protein. Myosin II 20- and 17-kD light chains are visible with samples of lower dilution. The remainder of the gel was transferred to nitrocellulose and probed with either anti-brush border myosin II antiserum (lanes *4–6*) or affinity-purified antimyosin I IgG (lanes *7–9*). Note that a brush border myosin II cross-reactive species at ∼100 kD is distinguishable from the 110-kD protein in these blots. Digestion of brush border myosin II with papain (Sellers et al., 1988) in the presence of actin was used to separate myosin II S1 and rod subdomains (lanes *11, 14*). Comparison with purified myosin I (lanes *10, 13*) and brush border myosin II before digestion (lanes *11, 14*) by Coomassie blue staining (lanes *10–12*) or transfer to nitrocellulose and incubation with anti-brush border myosin II antiserum (lanes *13–15*) indicates that myosin II S1 in the myosin I preparation would have been detected by the antiserum.
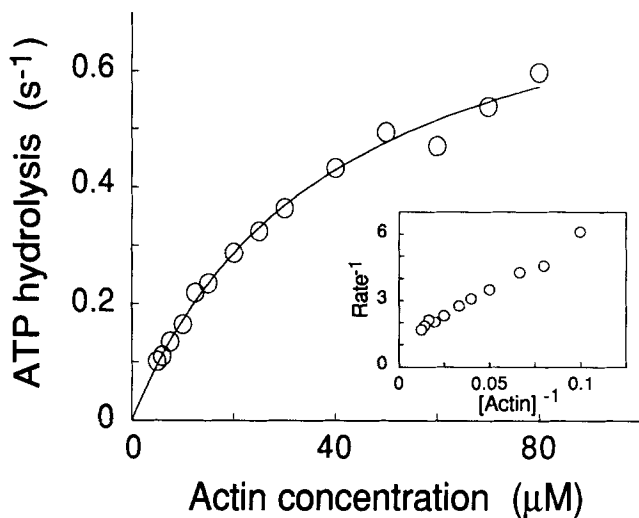

Figure 2. Myosin I actin-activated MgATPase activity. Assays for the ATPase activity of myosin I were performed in the presence or absence of actin. The actin-independent ATPase activity was 0.02 s^{-1}, which was subtracted from all measurements in the presence of actin. Rates of MgATP hydrolysis for actin concentrations ranging from one-fourth to twice the calculated K_m were included in a double-reciprocal plot of the kinetics (*inset*), which shows an actin-activated K_m of 40 μM and V_{max} of 0.86 s^{-1}. Similar results were obtained for different preparations of myosin I, with K_ms ranging from 20–40 μM.

this point were equivalent (Fig. 4 *c*), but below this threshhold the reliability of rate measurements decreased owing to Brownian motion of filament ends. This minimum enzyme requirement is similar to that found for platelet myosin (S. Umemoto and J. Sellers, personal communication). The ionic strength of the motility buffer did not greatly influence rate of motility (Fig. 4 *d*).

The effect of temperature on motility was assayed in temperature steps 15–50°C (Fig. 4 *e*). Over this range, the rate of motility increased with temperature 0.014–0.165 μm/s. A plot of the log of motility rate versus inverse temperature in Kelvin is consistent with a linear relationship (Fig. 4 *e*, *inset*). An increase in motility with temperature is also exhibited by myosin II, but the increase is biphasic (Sheetz et al., 1984). The ATP concentration required for the maximal rate of myosin I motility was also determined (Fig. 4 *f*). Motility saturates at ∼100 μM ATP with half-maximal activity at ∼60 μM ATP, similar to results obtained for myosin II (Sheetz et al., 1984).

Tropomyosin binding to actin activates the motility and ATPase activity of smooth muscle myosin II approximately twofold, the increase in ATPase activity due to an increase in V_{max} (Umemoto et al., 1989). In contrast, brush border myosin I motility is inhibited by tropomyosin: addition of 85 nM turkey gizzard smooth muscle tropomyosin in the fluorescent actin wash of the flow cell drastically reduced both the number and the motility of actin filaments bound to
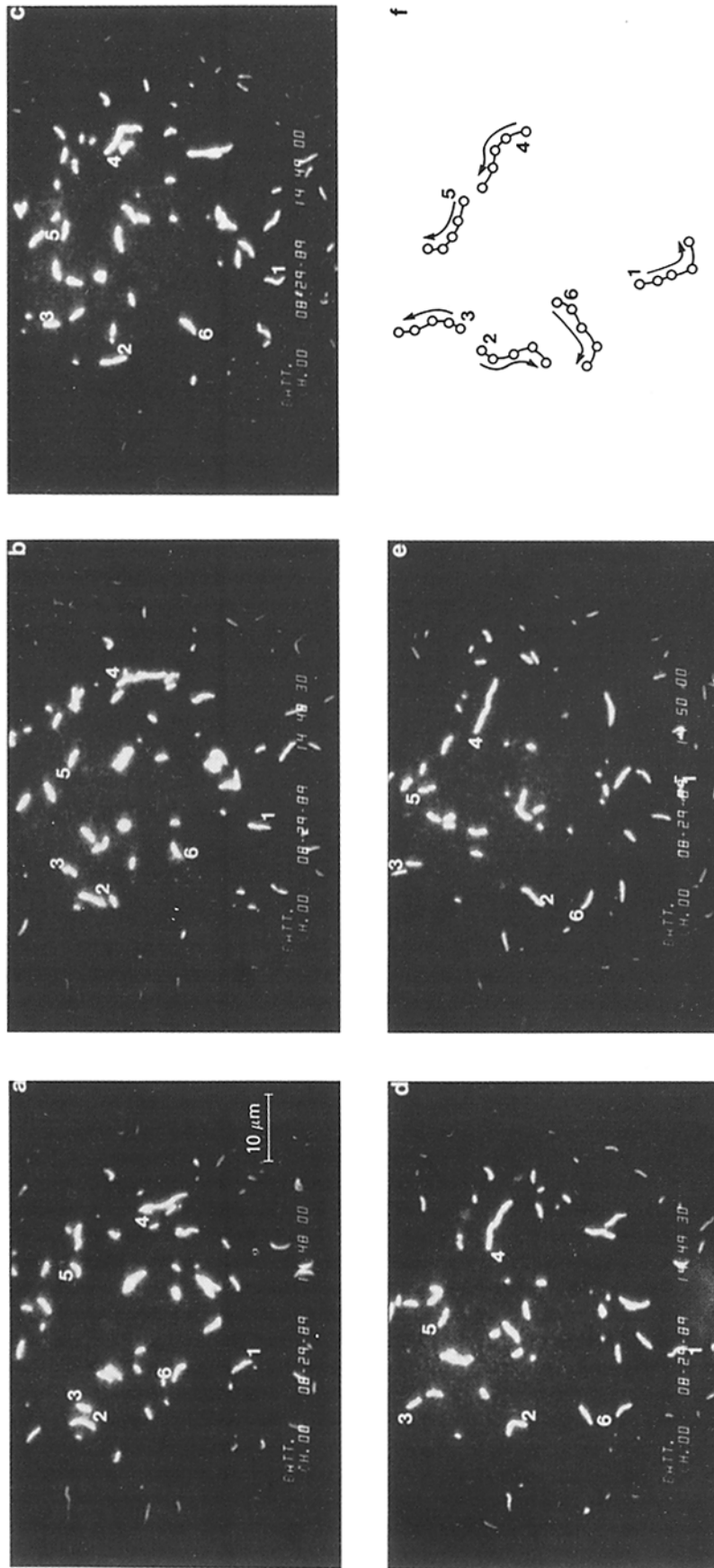
Figure 3. Translocation of actin filaments by brush border myosin I. Movement of fluorescently labeled actin filaments by immobilized myosin I was observed as described in Materials and Methods. Images of a single microscope field continuously recorded on videotape were photographed at 30-s intervals (*a–e*). Filament movement across the field occurs along a track of myosin heads. The progress of the six filaments numbered in the panels above is displayed in the final panel (*f*).
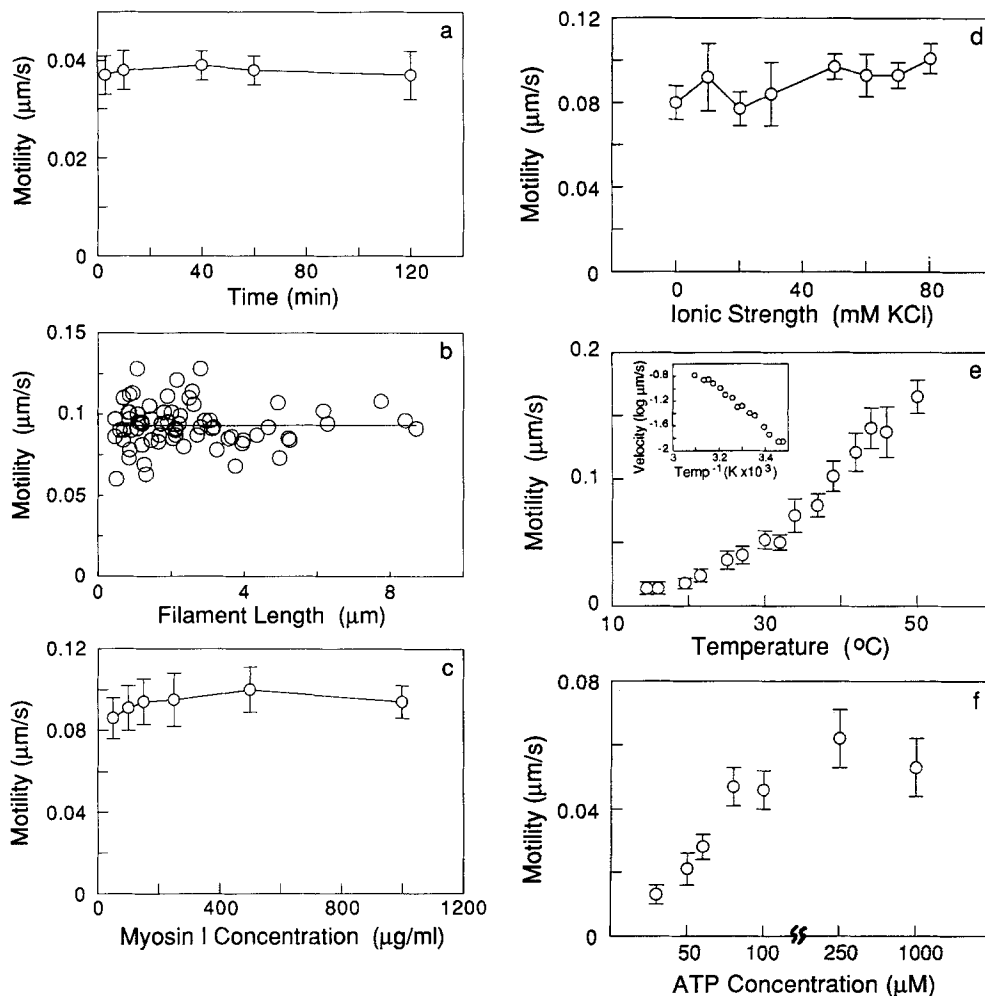
Figure 4. Properties of brush border myosin I-mediated motility. Filament movement was quantitated as described in Materials and Methods to determine the dependence of the rate of motility on (*a*) time of incubation, (*b*) actin filament length, (*c*) dilution of myosin I, (*d*) ionic strength, (*e*) temperature, and (*f*) concentration of ATP. In *a*, a single coverslip in pCa 9 motility buffer was kept at 25°C and examined for 3-min intervals at each time point. In *b*, the horizontal line represents the mean velocity of the 69 filaments quantitated in pCa 9 motility buffer in the figure (0.092 μm/s). In *c*, 25 μl of myosin I ranging in concentration from 50 μg/ml to 1 mg/ml was added to the flow cell, followed in exactly 2 min by washes with BSA solution, actin solution, and motility buffer. In *d*, the KCl concentration of the motility buffer was varied, maintaining constant pH. In *e*, a single coverslip was examined for 3 min at each temperature after equilibration of the slide in fresh, pH-adjusted motility buffer. The increase in rate of motility with temperature was fully reversible: rates measured while increasing temperature were the same as rates measured while decreasing temperature from a previous measurement. A plot of log of actin velocity versus inverse temperature in Kelvin is consistent with a linear relationship (*inset*). In *f*, pCa 9 motility buffer varying only in ATP concentration was added to coverslips that were identically coated with myosin.

the coverslip by myosin I. The same effect was produced by perfusion of the coverslip with tropomyosin after initiation of motility. This effect is most likely the result of a decreased affinity of myosin I for the actin–tropomyosin complex in the presence of MgATP relative to its affinity for actin alone. To verify that inhibition of motility results from binding of tropomyosin to actin in these experiments, rather than its binding to myosin I, we centrifuged a mixture of actin–tropomyosin–myosin I in the presence of MgATP. All tropomyosin pelleted with actin while the myosin I remained in solution in the supernatant (data not shown). Excess tropomyosin was required for effective inhibition of motility, probably due to the requirement for saturation of the actin before inhibition can be observed. Above the tropomyosin concentration required for saturation, we did not observe any variation of inhibition of motility with tropomyosin concentration. Quantitation of the rate of translocation in the presence of tropomyosin indicated that motility was completely inhibited, within the error of the measurements.

Regulation of Motility and ATPase Activity by Calcium

Coluccio and Bretscher (1987) reported that calcium causes calmodulin to dissociate from the 110-kD protein. The presence of calcium was also found to alter the behavior of myosin I during the chromatography steps of our purification procedure, suggesting a calcium-dependent change in the conformation of the complex. In motility assays containing 0.1 mM calcium, approximately the same number of actin filaments attached to the enzyme-coated coverslip before the addition of ATP as did in the absence of calcium. In the presence of ATP, however, although filaments were seen to bind and be released from the coverslip, indicating that myosin I in calcium can still bind actin, only rarely did translocation occur.

To investigate more carefully the relationship between calcium and motility, we measured the rate of filament movement in buffers of specific free calcium concentration over the 1-nM to 0.1-mM range. For two separate preparations of myosin I, we measured a 40% increase in the rate of actin translocation from pCa 9 to pCa 5 and an abrupt inhibition of motility at pCa 4 (Fig. 5 *a*). Complete inhibition of motility also occurred if 0.1 mM calcium was rinsed through the flow cell after the binding of myosin I but before addition of actin, assaying motility in buffer with either calcium or

EGTA. Since inhibition can occur with incubation of myosin I in calcium in the absence of actin, and since inhibition is not alleviated in the subsequent presence of EGTA, calcium causes an irreversible structural change within the myosin I complex.

To determine if calcium also modulated the rate of ATP hydrolysis mediated by myosin I, we measured the ATPase activity of the complex in the same calcium buffers used in the motility assays. Total ATP hydrolysis in the presence of 20 μM actin generally increased with calcium (Fig. 5 b). To investigate the relationship between rate of motility and Mg-ATP hydrolysis, we determined what proportion of total ATPase activity was contributed by actin-dependent versus actin-independent ATPase activities. Fig. 5 b shows that the actin-independent ATPase activity increased linearly as calcium increased above 1 μM concentration, while below this concentration it remained constant. Control experiments (not shown) showed that the intrinsic ATPase activity of actin was constant over this range of calcium concentration, so the effect of calcium on actin-dependent MgATPase activity was determined by subtraction of actin-independent ATPase activity from total ATPase activity (Fig. 5 c). The maximal rate of actin-dependent ATP hydrolysis occurred at pCa 7, increased twofold over the rate at pCa <5, where motility was inhibited, or pCa ≥8. This pattern of increase and decrease in actin-dependent ATPase activity with calcium was measured in several different preparations of myosin I, and is therefore not likely to be the result of degradation or contamination. The pCa buffers are significantly higher in ionic strength than the buffer used for determination of kinetic constants in Fig. 2. The increased ionic strength is probably responsible for the reduced actin activation of ATPase activity at 20 μM actin in this experiment. In support of this conclusion, MgATPase activity in pCa buffers with 60 μM actin is activated 20-fold by actin, consistent with an increased K_m (Table I; see subsequent Results). Increased ionic strength has been shown to increase the K_m of actin activation for

several smooth muscle and cytoplasmic myosins (Sellers et al., 1988).

Calcium-dependent Structure of the Myosin I Complex

Because calcium modulated both myosin I motility and ATPase activity, we analyzed its effect on the structure of the complex using velocity sedimentation. Glycerol gradients of 15–30% were used to resolve myosin I monomers from dimers, as well as calmodulin bound to or dissociated from the myosin complex. When centrifuged with or in parallel to standards, in calcium or in EGTA, the 110-kD protein and calmodulin cosedimented with a molecular mass near 150 kD. These results indicate that in solution, the 110-kD heavy chain does not self-associate. In EGTA, all calmodulin remained associated with the complex. In 0.1 mM calcium, however, a fraction of the calmodulin sedimented at ~20 kD, dissociated from the complex.

To determine the structure of the complex under the conditions tested for motility, we sedimented myosin I through glycerol gradients made with the free calcium buffers from pCa 9 to pCa 3. All calmodulin remained associated with the complex during sedimentation at pCa ≥ 7. At higher calcium concentrations, SDS-PAGE (Fig. 6) shows that dissociated calmodulin was detected in increasing amounts. Dissociation appeared maximal at pCa 4. The precision of the method did not allow us to determine the exact number of calmodulin subunits dissociated per complex.

Calcium Inhibition of Motility and ATPase Activity Is Reversed by Addition of Calmodulin

Because calcium inhibition of myosin I activities correlated with the loss of a subset of calmodulin from the complex, we tested the ability of exogenous calmodulin to restore activity to the calcium-inhibited enzyme. Addition of exogenous calmodulin at 0.5 or 5 μM to the motility buffer did not significantly alter the rate of motility mediated by the complex under any pCa condition, but addition of 25 μM calmodulin to the motility buffer resulted in continued motility at pCa 4. Coverslips rinsed in 1 mM calcium and observed in calcium motility buffer containing 25 μM calmodulin moved filaments at 0.061 ± 0.006 μm/s. Coverslips rinsed
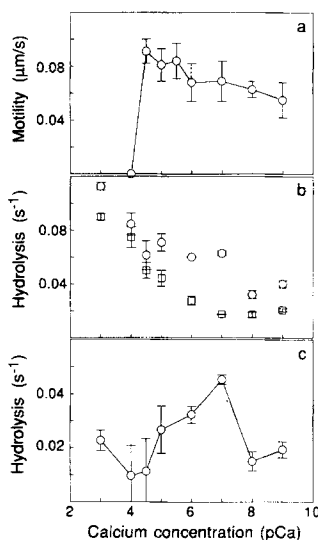

Figure 5. Calcium regulation of motility and ATPase activity. (a) Dependence of rate of motility on pCa. All motility measurements in this pCa set were from the same myosin I sample; other samples gave similar results. (b) Dependence of actin-stimulated ATPase activity (total ATPase activity, circles) and actin-independent ATPase activity (squares) on pCa. The two measurements differ only in the addition of 20 μM actin or an equal volume of identical buffer not containing actin. The rates shown were all measured using the same myosin I sample; other samples give similar results. (c) Dependence of Mg-ATPase activity on pCa. Calcium regulation of MgATPase activity was determined by subtraction of actin-independent ATPase activity from total ATPase activity using the rates in b (both values for pCa 5 were included in the calculations).

Table I. Effects of Calcium and Calmodulin on Myosin I ATPase Activity

	Actin		ATPase activity	Error
	(μm)		(s^{-1})	(s^{-1})
	0		0.047	0.005
pCa 9	20	−CaM	0.210	0.049
		+CaM	0.236	0.032
	60	−CaM	0.912	0.083
		+CaM	0.889	0.149
	0		0.403	0.021
pCa 3	20	−CaM	0.689	0.113
		+CaM	0.854	0.066
	60	−CaM	0.597	0.077
		+CaM	1.216	0.060

All rates and errors were calculated from the combined 10-, 20-, 30-, and 40-min time points of two separate reactions. Calmodulin (final concentration ~10 μM) or an equal volume of buffer was added to each sample.
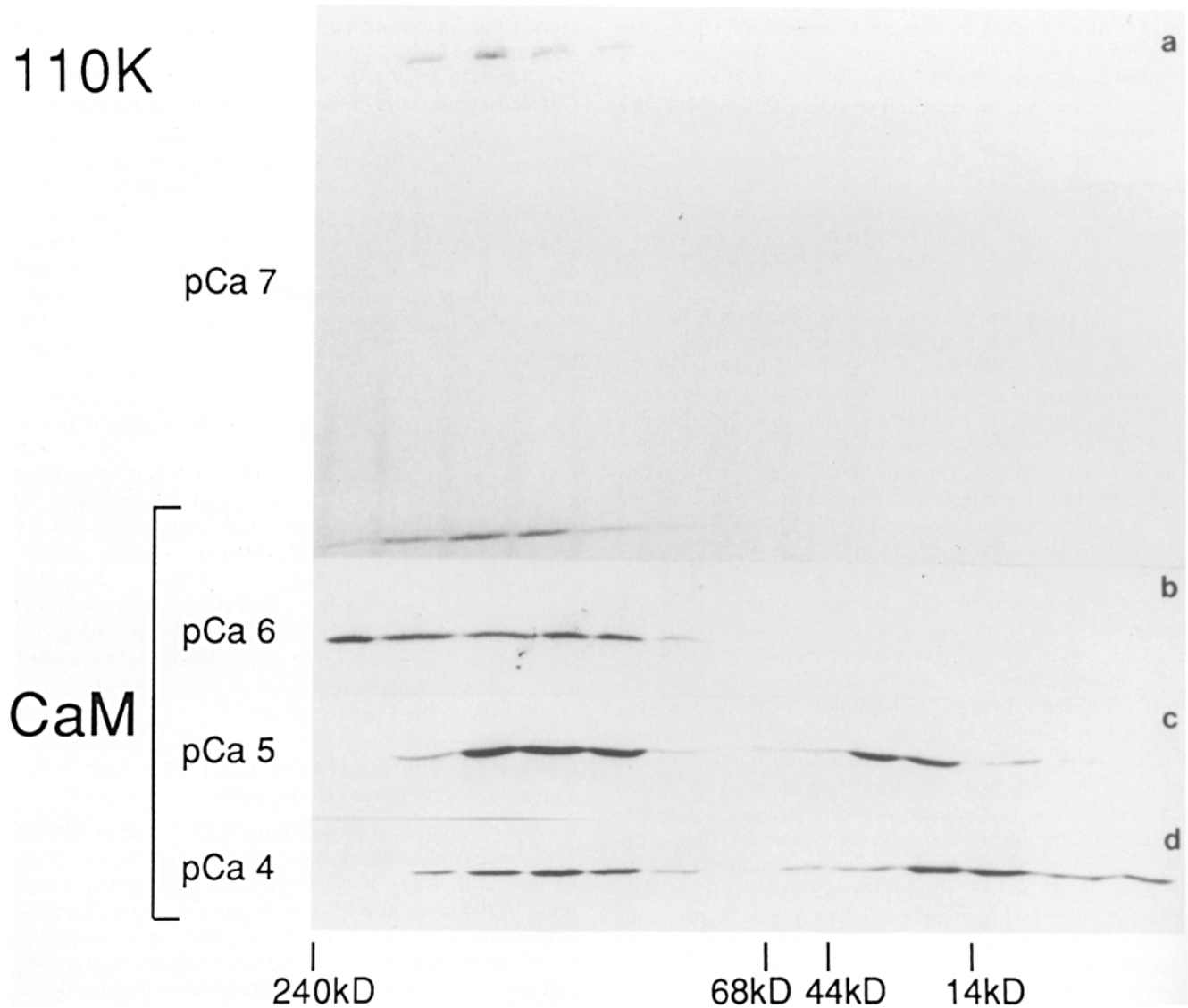
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Figure 6. Subunit association in the myosin I complex. Glycerol gradients of a total volume of 4.45 ml were separated dropwise into ~200-µl fractions and analyzed by SDS-PAGE after addition of 5× sample buffer. In solution under all conditions tested, for example, at pCa 7 (*a*), the 110-kD protein and at least a subset of calmodulin subunits sedimented midway between the 240- and 68-kD markers. Although all calmodulin cosedimented with the myosin I complex at pCa 7, its dissociation increased with increasing calcium (*b–d*); detectable in some samples at pCa 6 and reaching maximal dissociation apparent in the gradients at pCa 4. In *b–d*, only the calmodulin region of each gel is shown, since the 110-kD protein migrated similarly in each sample.

or observed in 1 mM calcium and not rescued by calmodulin demonstrated no motility. Parallel coverslips not rinsed in calcium, washed only in EGTA, moved filaments at 0.084 ± 0.013 µm/s. Also, myosin I first washed in 1 mM calcium, next washed in EGTA buffer containing 25 µM calmodulin, and then observed in EGTA motility buffer without calmodulin, translocated actin filaments at 0.069 ± 0.017 µm/s. For all coverslips rescued from calcium inhibition, >95% of filaments in a field moved. This suggests that calcium inhibition of motility is the result of dissociation of calmodulin from the complex.

The effect of exogenous calmodulin on the ATPase activity of the complex was assayed at pCa 3 and pCa 9, at both 20 and 60 µM actin (Table I). At pCa 9, addition of 10 µM calmodulin has no effect on myosin I ATPase activity at either 20 or 60 µM actin. Actin activation of MgATP hydrolysis increases from 4-fold at 20 µM to 20-fold at 60 µM actin as would be expected from the actin activation of myosin I ATPase activity (Fig. 2). At pCa 3, however, in the absence of exogenous calmodulin, the rate of MgATP hydrolysis at both actin concentrations is the same. Actin does not appear to activate MgATPase activity above a basal level. In contrast, in the presence of calmodulin, MgATPase activity increases with actin concentration. This rescue of actin-activated ATPase activity is not due to a reduction in calcium concentration with addition of calmodulin, as the calcium concentration (1 mM) is in vast excess of calmodulin (10 µM).

Discussion

Myosins I and II Share Common Molecular Mechanisms

Despite the structural differences between myosins I and II,

our results indicate that their enzymatic properties, at least for brush border myosin I and nonmuscle myosin II, are remarkably similar. We have shown that brush border myosin I MgATPase activity has the actin dependence and simple Michaelis-Menten kinetics of actin activation characteristic of myosin II MgATPase activity. The intrinsic, actin-independent MgATPase activity of the 110-kD complex in our preparation was lower and the actin activation greater than in previous studies, which have reported a calcium-stimulated, actin-independent rate of 50–100 nmol/min/mg (0.13–0.27 s^{-1}) and two to threefold stimulation by actin. The difference in results could be explained by loss of calmodulin subunits in previous preparations, as the actin-independent rate and low actin activation resemble the activities of the partially calmodulin-depleted complex (Table I, pCa 3). Also, we have demonstrated that the rate of myosin I motility is equal to the rate of cytoplasmic myosin II motility, ∼0.08 μm/s. We have determined that myosin I's rate of motility is independent of the number of actin–myosin interactions and dependent on temperature and an ATP concentration similar to that required for myosin II motility. These common properties suggest that the two enzymes have conserved structures important in ATP hydrolysis, actin binding, and molecular transduction of force.

It is not surprising that a myosin I lacking the coiled-coil tail of myosin II could share the same mechanism of motility. The motility of *Acanthamoeba* myosin I and of proteolytic fragments of myosin II lacking the coiled-coil tail has already been documented (Albanesi et al., 1985; Toyoshima et al., 1987). The motility mediated by single-headed myosin (Harada et al., 1987) and by myosin S1 (Toyoshima et al., 1987) indicates that in vitro only a single actin-binding site per molecule is necessary for translocation. In vivo, however, brush border myosin I represents the first myosin molecule with a single actin-binding site. Although the structure of *Acanthamoeba* myosin I indicates that it may generate movement by sliding one actin filament relative to a second filament bound at its carboxy-terminal, ATP-independent actin-binding site (Lynch et al., 1986), no equivalent brush border myosin I anchor is known.

Although the cytoplasmic myosins I and II may be indistinguishable mechanistically, purified brush border myosin I is a more stable actin motor in vitro. The rate and regulation of brush border myosin I motility are unaffected by the age of the purified complex for at least 5 wk, while in our experience myosin II motility becomes difficult to quantitate after a period of days. On the coverslip, myosin I supports motility for greater than two hours without change in actin velocity. Examination of the bovine brush border myosin I sequence (Hoshimaru and Nakanishi, 1987) reveals a possible explanation for this stability: myosin I lacks the two reactive thiol groups common to many myosin II heavy chains (Warrick and Spudich, 1987). Alternately, the stability difference could result from other structural differences or from a difference in purity, with reduced proteolysis of a more highly purified myosin I. The enhanced stability of brush border myosin I activity in vitro and its native single-headed structure suggest that this enzyme will provide an excellent model for future study of the molecular mechanism of motility.

The brush border myosin I motility that we describe contrasts with that observed by Mooseker and Coleman (1989).

Measuring the movement of Latex beads covalently bound with purified myosin I on a substrate of *Nitella* actin cables, they reported an average rate of motility of 0.008 μm/s that decreased within an hour to zero. The difference in rate between their results and ours is surprising, given the equivalent rates of motility of other myosins in the two assays (S. Umemoto and J. Sellers, personal communication). In a subsequent study (Mooseker et al., 1989), myosin I-containing microvillar membrane disks were coupled to beads. The investigators observed a rate of motility fourfold higher than previously observed for myosin I alone.

In opposition to our findings, Mooseker and Coleman (1989) also reported that the motility of the purified complex requires calcium. The difference in results could be due to (*a*) the state of phosphorylation (or other regulation) of the purified complex; (*b*) the degree of purity of the sample, (*c*) loss of calmodulin light chains; or (*d*) alteration of the complex due to direct bead coupling. The calcium independence of membrane disk motility (Mooseker et al., 1989) and the unpublished observation of Mooseker and Coleman of the calcium-independent movement of beads coupled to purified myosin I via an mAb (Mooseker and Coleman, 1989), would seem to indicate that calcium independence was conferred by an associated protein or an induced conformational change. Our results indicate that modification of myosin I structure by an associated protein is not necessary for calcium-independent translocation in vitro.

Regulation of the Motility, ATPase Activity, and Structure of Brush Border Myosin I by Calcium

Myosin I treated with high calcium buffer (pCa 4 or 3) is unable to translocate actin filaments and hydrolyzes MgATP at a basal rate not stimulated by increasing actin. This inhibition in calcium is correlated with the partial dissociation of calmodulin from the myosin I complex. We have demonstrated that motility and actin-activated MgATPase activity can be completely restored by addition of exogenous calmodulin, even in 1 mM calcium. These findings suggest that partial dissociation of calmodulin is entirely responsible for the calcium inhibition of brush border myosin I motility and actin-activated ATPase activity. At least in vitro, calmodulin dissociation can be thought of as a reversible "on-off" switch for the mechanochemical activities of myosin I.

The apparent requirement for nonphysiological calcium levels (pCa 4) to inhibit motility, and the sharp transition to inhibition at pCa 4, are the consequence of two phenomena: a gradual reduction in the amount of intact complex from pCa 7 to 4 (Fig. 6), and the concentration independence of motility rate (Fig. 4 c). Unlike a rigor head, the nonmotile complex can bind and release actin in the presence of ATP, so although it does not translocate actin it does not act as an internal load to translocation by the active complex. As the amount of partially-dissociated complex increases from pCa 7 to 4, its lack of activity is not apparent: the intact complex remaining on the coverslip can still translocate actin. Not until pCa 4, when sedimentation of the complex in glycerol gradients indicates that dissociation is maximal, does the assay measure a lack of motility. The dissociation of calmodulin from the complex in calcium as low as micromolar is evident from the glycerol gradients and from the inhibition of MgATPase activity beginning at pCa 6. The gradualness of

calmodulin dissociation may be exclusively an in vitro phenomena, due to solution conditions. It does not imply a similar gradual dissociation in vivo, or even that calmodulin dissociates from the complex in vivo, but it does indicate that calmodulin can regulate the activity of the myosin I complex.

We have shown that partial dissociation of calmodulin from myosin I inhibits motility and actin activation of Mg-ATPase activity. These inhibitions are strikingly similar to the inhibition of scallop myosin II after reversible dissociation of regulatory light chain: dissociation drastically reduces motility and deregulates ATP hydrolysis (Vale et al., 1984). If partial loss of calmodulin from brush border myosin I is producing the same inactivation as removal of light chain from myosin II, this suggests that of the three to four calmodulin molecules complexed to the 110-kD protein, perhaps the subset that dissociates in calcium in vitro is acting as light chain for myosin I. The additional calmodulin remaining associated with the 110-kD protein could have a separate function, thus explaining the abnormally large proportion of low molecular weight subunits in the brush border myosin I complex compared with other myosin molecules.

It appears from these results that light chain can regulate the coordination of heavy chain activities. Although inhibition of actin activation of ATPase activity appears to be coupled with inhibition of motility, a low unregulated rate of MgATPase activity can continue in the absence of motility. The transition between translocation-coupled MgATPase activity and translocation-uncoupled MgATPase activity depends, at least in vitro, on light chain association.

Myosin I Motility In Vivo

Movement and polarity of cells, translocation of organelles, and localization of cellular compartments, proteins, and possibly mRNA are all thought to involve or be dependent upon the cytoskeleton of the cell. Recently, the discovery of several microtubule motors with distinct structure and motility has suggested that different motors will direct different kinds of microtubule-dependent traffic in the cell. Microfilament-dependent motility may also employ specific motors for specific jobs, but only two classes of motor molecules are known, myosins I and II. The differences in structure, regulation, and localization of these two motors suggest that they serve complementary functions.

Although myosin II has been implicated in muscle contraction, cytokinesis, and cell shape changes, the persistence of motility, polarity, and formation of membrane projections in *Dictyostelium* cells lacking myosin II by antisense mRNA disruption (Knecht and Loomis, 1987) or gene replacement (De Lozanne and Spudich, 1987) suggests that another actin motor, perhaps myosin I, may be involved in these events. In support of this suggestion, myosin I localizes to the leading edge of motile *Dictyostelium* amoeba (Fukui et al., 1989). In both *Acanthamoeba* and *Dictyostelium*, several distinct myosin I proteins exist in each cell, the *Acanthamoeba* myosin I isoforms demonstrated to have distinct properties (Korn and Hammer, 1988; Korn et al., 1988; Titus et al., 1989).

In contrast to *Acanthamoeba* and *Dictyostelium*, the intestinal epithelial cells containing brush border myosin I are highly differentiated and are joined tightly to each other and the basement membrane. The cells do not move relative to

one another, and the microvillar actin core does not appear to move relative to the cell surface (Matsudaira and Burgess, 1982; Burgess and Prum, 1982). What role could an actin motor serve in nonmotile microvilli? Although morphologically microvilli appear static, the components of the cytoskeleton are individually dynamic: rates of protein and membrane turnover in brush border are high (Stidwill et al., 1984). Because myosin I may be excluded from tropomyosin-containing actin complexes such as the rootlets of microvilli, stress fibers, the contractile ring, and the circumferential belt, and because of the intrinsic polarity of microvillar actin filaments with respect to the membrane, all myosin I-mediated vesicle transport would be directed to the apical cell surface. In other cells, vesicle movement or movement of membrane-associated actin filaments mediated by myosin I could be involved in directed cell growth, cytoplasmic streaming, or formation of membrane projections. We hope that examination of other vertebrate myosin Is will contribute to a better understanding of the role of myosin I in the cell.

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