The 110-kD Protein–Calmodulin Complex of the Intestinal Microvillus Is an Actin-activated MgATPase

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Abstract. The microvillus 110-kD protein-calmodulin complex (designated 110K-CM) shares several properties with all myosins. In addition to its well-defined ATP-dependent binding interaction with F-actin, 110K-CM is an ATPase with diagnostically myosin-like divalent cation sensitivity. It exhibits maximum enzymatic activity in the presence of K^+ and EDTA (0.24 µmol P_i/mg per min) or in the presence of Ca^{++} (0.40 µmol P_i/mg per min) and significantly less activity in physiological ionic conditions of salt and Mg^{++} (0.04 µmol P_i/mg per min). This MgATPase is activated by F-actin in an actin concentration–dependent manner (up to 2.5–3.5-fold). The specific MgATPase activity of 110K-CM is also enhanced by the addition of 5–10 µM Ca^{++}, but in the isolated complex, there is often also a decrease in the extent of actin activation in this range of free Ca^{++}. Actin activation is maintained, however, in samples with exogenously added calmodulin; under these conditions, there is an approximately sevenfold stimulation of 110K-CM's enzymatic activity in the presence of 5–10 µM Ca^{++} and actin. 110K-CM is relatively indiscriminant in its nucleoside triphosphate specificity; in addition to ATP, GTP, CTP, UTP, and ITP are all hydrolyzed by the complex in the presence of either Mg^{++} or Ca^{++}. Neither AMP nor the phosphatase substrate *p*-nitrophenyl phosphate are substrates for the enzymatic activity. The pH optimum for CaATPase activity is 6.0–7.5; maximum actin activation of MgATPase occurs over a broad pH range of 6.5–8.5. Finally, like myosins, purified 110K-CM cross-links actin filaments into loosely ordered aggregates in the absence of ATP. Collectively these data support the proposal of Collins and Borysenko (1984, *J. Biol. Chem.*, 259:14128–14135) that the 110K-CM complex is functionally analogous to the mechanoenzyme myosin.

The actin filament bundle within each microvillus of the intestinal epithelial brush border is linked laterally to the plasma membrane by a helical (Matsudaira and Burgess, 1982*a*) array of cross-bridges (Mukherjee and Staehelin, 1971; Mooseker and Tilney, 1975). These spirally arranged bridges are thought to be comprised, at least in part, of a complex of calmodulin (CM)[1] and a subunit of 110,000 (designated 110K) (for review see Mooseker, 1985). Studies using both demembranated and membrane-intact microvilli (Matsudaira and Burgess, 1979; Verner and Bretscher, 1983) and brush borders (Howe and Mooseker, 1983) have demonstrated that the lateral bridges are dissociated from the cytoskeleton by treatment with ATP. Such ATP treatment also solubilizes the 110K-CM complex. The most compelling evidence that 110K-CM comprises the lateral bridge is the finding that, like the bridge, purified 110K-CM binds to F-actin in an ATP-dependent manner (Howe and Mooseker, 1983; Collins and Borysenko, 1984; Verner and Bretscher, 1985). These studies have helped to clarify the molecular basis for the interaction of 110K-CM with the microvillar actin filament bundle; however, the nature of its association with the plasma membrane remains unresolved (for discussion see Conzelman and Mooseker, 1986; Mooseker, 1985).

Aside from the likely structural role that 110K-CM plays in tethering of the microvillus core to the membrane, the function of the complex in the brush border is unknown. Since the intestinal epithelium is the organism's primary site of vitamin D–dependent calcium uptake (for review see Bikle et al., 1981; Bronner et al., 1986), 110K-CM may provide a stable calcium-buffering potential for the cell (e.g., see Glenney and Glenney, 1985). The 110K-associated calmodulin may also have the capability to activate Ca^{++} and calmodulin-dependent enzymes, thereby providing structurally localized regulation of such enzymes. Finally, as first suggested by the work of Collins and Borysenko (1984), 110K-CM may be a myosin-like enzyme. This hypothesis is based on the observation that, in addition to its ATP-dependent binding interaction with actin, 110K-CM exhibits relatively high ATPase activity in solutions containing K^+ and EDTA or Ca^{++} and comparatively low activity in the presence of physiological Mg^{++} and salt concentrations. Such activities are consistent with the enzymatic properties of all myosins (Pollard, 1982). The work reported here is a detailed characterization of the enzymatic properties of the 110K-CM complex, including the determination of substrate specificity, pH

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[1] *Abbreviations used in this paper:* CM, calmodulin; HAP, hydroxylapatite.

optima, and effects of the CM antagonist trifluoperazine and sodium orthovanadate on its ATPase activity. These studies confirm and extend the previous observations of Collins and Borysenko (1984) regarding the divalent cation sensitivity of 110K-CM's ATPase activity and most importantly also show that, like myosin, the MgATPase of 110K-CM is activated by the addition of F-actin. Similar levels of actin activation for 110K-CM have been reported in preliminary form by Swanljung-Collins et al. (1986). This actin activation at physiological ionic conditions provides critical support for the notion that 110K-CM may be involved in mechanochemical coupling in vivo.

Materials and Methods

All reagents were obtained from Sigma Chemical Co., St. Louis, MO unless otherwise noted.

Purification of 110K-CM

Brush borders were fractionated from chicken intestinal epithelial cells by the method of Mooseker and Howe (1982) with the modifications of Keller and Mooseker (1982). Diisopropyl fluorophosphate (0.34 g/liter), aprotinin (30–60 trypsin inhibitor units/liter), and phenylmethylsulfonyl fluoride (PMSF) (0.2 mM) were added to the initial homogenization buffer to minimize proteolytic activity. All subsequent solutions contained aprotinin (10–20 trypsin inhibitor units/liter) and PMSF (0.2 mM).

110K-CM–enriched fractions were obtained by slight modification of the method of Conzelman and Mooseker (1986). In brief, brush borders were resuspended in 10–15 vol of 5 mM ATP in solution I (75 mM KCl, 10 mM imidazole-Cl, 1 mM EGTA, 0.1 mM $MgCl_2$, 0.01% NaN_3, 0.2 mM dithiothreitol [DTT] [Bretscher, 1981]) and incubated 10 min on ice. The 27,000 *g* supernatant was loaded onto an 8–10-ml hydroxylapatite (HAP) (Bio-Rad Laboratories, Richmond, CA) column (2.5-cm diameter), equilibrated in Buffer A (0.3 M KCl, 10 mM imidazole-Cl, 0.1 mM $MgCl_2$, 1 mM EGTA. 0.02% NaN_3) with 75 mM potassium phosphate (pH 7.0). Proteins were eluted with a 200-ml linear 75–230-mM potassium phosphate gradient in Buffer A. Peak fractions, free of any brush border myosin, eluting at phosphate concentrations of 135–175 mM, were pooled and dialyzed for 12–18 h in solution B (75 mM KCl, 20 mM imidazole-Cl [pH 7.5], 0.1 mM $MgCl_2$, 0.1 mM EGTA, 0.02% NaN_3). 110K-CM was further fractionated from contaminating polypeptides by passage over a 2-ml (1.5-cm diameter) Q-Sepharose column (Pharmacia Fine Chemicals, Piscataway, NJ), equilibrated in solution B. Elution with a 75–600-mM KCl (final concentrations) gradient in solution B yielded fractions containing purified 110K-CM at salt concentrations of 350–500 mM. This pooled peak was dialyzed into solution C (75 mM KCl, 20 mM imidazole-Cl [pH 7.5], 0.1 mM $MgCl_2$, 0.02% NaN_3, 0.2 mM DTT) before assay; it varied in final protein concentration from 30–90 µg/ml. Attempts to concentrate the complex by a variety of standard techniques all resulted in precipitation and/or loss of protein.

Assays for Enzymatic Activity

ATPase activity was assayed by the method of Taussky and Shorr (1953). All samples were incubated at 35°C, and assays were initiated by the addition of 2 mM K-ATP. Ca and K-EDTA ATPase activities were measured over 20–30-min intervals; MgATPase assays were conducted for 60 min. The release of inorganic phosphate was determined to be linear over the time course of these assays (not shown). The final conditions used were as follows: (for MgATPase) 75 mM KCl, 20 mM imidazole-Cl (pH 6.85 at 35°C), 5 mM $MgCl_2$, 5 mM EGTA, 0.02% NaN_3, 0.2 mM DTT; (for CaATPase) 75 mM KCl, 20 mM imidazole-Cl (pH 6.85 at 35°C), 0.1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.02% NaN_3, 0.2 mM DTT; (for K-EDTA-ATPase) 0.575 M KCl, 20 mM imidazole-Cl (pH 6.85 at 35°C), 2 mM EDTA, 0.02% NaN_3, 0.2 mM DTT. To test the effect of F-actin on the enzymatic activity of 110K-CM, actin was added to the assay mixtures either from a concentrated stock of F-actin, prepolymerized in solution C, or of G-actin, allowed to reach steady state at room temperature before the start of the assay. The final actin concentration used ranged from 0.3 to 0.9 mg/ml; the concentration for each assay is noted in the figure legends. The actin concentration dependence of MgATPase activity was assessed by addition of actin over a range of concentrations from 0.1 to 60 µM. Phalloidin (Boehringer Mannheim Biochemicals, Indianapolis, IN) (2:1 molar ratio with actin) was added to the most dilute stocks to stabilize the actin as filaments. The ATPase activity of F-actin at each of these concentrations was measured under these conditions, and its contribution was subtracted from the corresponding actin-activated activity of the 110K-CM.

The substrate specificity of 110K-CM was examined by substituting other nucleoside triphosphates (NTPs) (specifically GTP, CTP, UTP, and ITP), AMP, or *p*-nitrophenyl phosphate for ATP in the assay mixtures.

The effect of elevated monovalent salt on enzymatic activity was assessed by the addition of 0.5 M NaCl or KCl to the standard assay mixtures.

To determine the pH dependence of both CaATPase and MgATPase activities, the pH of the assay mixtures was titrated over a range of 5.5 to 8.5 (at 35°C) by the addition of 1 M buffer stocks (sodium acetate, pH 5.5; Pipes, pH 6.0–6.5; imidazole, pH 6.9; Tris, pH 7.2–8.6) to a final concentration of 50 mM; any differences in ionic strength were compensated by the addition of 1 M KCl to equivalent conductivities.

The effect of Ca^{++} on the MgATPase activity was assessed by the addition of Ca-EGTA buffers (ratios of 0:1, 0.65:1, 0.85:1, 0.95:1, and 1:1; final concentration of 5 mM EGTA in all samples) to the standard MgATPase assay mix in place of EGTA. This covers a range of free Ca^{++} from $<10^{-9}$ to 10^{-5} M (Portzehl et al., 1964). We also confirmed the capability of these buffers to stimulate the activity of Ca^{++}-CM–dependent enzymes using activation of gizzard myosin light chain kinase activity as a "bioassay." Gizzard myosin light chain kinase was purified (with the help of Dr. T. C. S. Keller) by the method of Guerriero et al. (1981), and its kinase activity was assayed as described by Keller et al. (1985). Samples were run on SDS-polyacrylamide gels, and myosin light chain phosphorylation was assessed by autoradiography. Under these ionic conditions, gizzard myosin light chain kinase was maximally activated by the 0.95:1 Ca-EGTA buffer used in these studies.

The effect of sodium orthovanadate (Fisher Scientific Co., Fair Lawn, NJ) on ATPase activity was assayed over a range of 1 to 250 µM.

Trifluoperazine (Smith Kline & French Laboratories, Philadelphia, PA) was added to the standard assays at concentrations from 20 to 50 µM. The inhibitory efficacy of the drug was tested on gizzard myosin light chain kinase activity; complete inhibition of light chain phosphorylation was obtained at 50 µM trifluoperazine.

110K-CM–Actin Cosedimentation

110K-CM was selectively fractionated from pooled HAP fractions in solution D (solution B with KCl added to a final concentration of 0.3 M) by the addition of F-actin to 0.1 mg/ml and centrifugation at 100,000 *g* in a Beckman SW51 rotor for 60 min onto a 60% sucrose cushion in solution B. Actin and sucrose were added to the supernatant to equivalent concentrations as the resuspended pellet. Both fractions were assayed for MgATPase and CaATPase activity. To assess filament cross-linking by 110K-CM, F-actin was added to 110K-CM in solution C ± 5 mM ATP to a final concentration of 0.2 mg/ml. After a 10-min incubation on ice, additional ATP or buffer was added. After an additional 10 min at 0°C, the samples were spun at 39,000 *g* for 30 min (modification of Matsudaira and Burgess, 1982*b*). The protein composition of the resulting supernatant and pellet fractions was assessed by SDS-PAGE. Similar preparations of F-actin and 110K-CM in the absence and presence of ATP were examined by electron microscopy of negatively stained samples and by darkfield light microscopy.

The approximate 110K-CM/actin ratio required to achieve apparent saturation of binding was determined by adding a range of F-actin (1–25 µg/ml; stabilized as filaments with a 1:1 molar ratio of phalloidin) to a fixed concentration of 110K-CM (50 µg/ml) in solution C. Aliquots of these preparations were negatively stained for electron microscopy as detailed below. The rest of each sample was centrifuged at 26 psi for 30 min in an airfuge (Beckman Instruments Inc., Palo Alto, CA). The relative distribution of the complex in pellet and supernatant fractions was assessed by SDS-PAGE; protein bands were visualized with silver stain (Morrissey, 1981). The actin concentration at which the 110K-CM was no longer exclusively pelleted was estimated to be a titration through the apparent saturation point for binding.

Microscopy

Mixtures of F-actin and 110K-CM identical to those described above for cosedimentation analysis were visualized by darkfield light microscopy and electron microscopy. Light micrographs were taken on Tri-X film using a Zeiss light microscope with darkfield condenser. Samples for electron microscopy were stained with 1% aqueous uranyl acetate on parlodion and carbon-coated grids and examined on a Zeiss 10CA electron microscope at an accelerating voltage of 80 kV.

Rotary replicas of 110K-CM and proteolytic fragments of myosin were prepared by the method of Heuser (1983).

Preparation of Other Proteins

Calmodulin was prepared from bovine testes by the method of Burgess et al. (1980) (graciously provided by T. Coleman and Dr. T. Shibayama). Actin was prepared from chicken breast muscle by the method of Spudich and Watt (1971). Myosin subfragment-1 was prepared from whole rabbit skeletal muscle fibers by digestion with papain (Cooke, 1972; kindly provided by Dr. R. Cooke). Heavy meromyosin was prepared by chymotrypic digestion of purified skeletal muscle myosin (Margossian and Lowey, 1982) by the method of Weeds and Taylor (1975) (kindly provided by Dr. J. Heuser).

Other Methods

Protein concentration of purified 110K-CM was measured using the BCA micro-protein determination assay (Pierce Chemical Co., Rockford, IL) using BSA as a standard. The concentration of BSA, rabbit muscle phosphorylase-a, calmodulin, and G-actin was measured by absorbance using extinction coefficients of 8.2 (at 280 nm; Morton, 1975), 11.8 (at 277 nm; Fasman, 1975), 2.0 (at 280 nm; Burgess et al., 1980), and 10.9 (at 280 nm; Margossian and Lowey, 1973), respectively.

SDS-PAGE (Laemmli, 1970) was performed using 5-16% linear gradient minigels (Matsudaira and Burgess, 1978). The molar ratio of 110K to calmodulin in the isolated complex was determined by quantitating the density of Coomassie Blue-stained gel bands (Fairbanks et al., 1971) relative to actin and phosphorylase-a (for 110K) and to purified calmodulin (for CM). The relative concentrations of 110K in column fraction was also measured by densitometry scans of stained gel bands, using a No. 1650 densitometer (Bio-Rad Laboratories). The area under curves was integrated using a planimeter.

Results

Purification of 110K-CM

Purified 110K-CM was prepared from ATP extracts of chicken brush borders by sequential HAP and ion-exchange (Q-Sepharose) chromatography (Fig. 1, *a* and *c*). The peak fractions purified by this method are free of the higher molecular mass polypeptide (~130 kD) present in trace amounts in the preparations of Howe and Mooseker (1983). Furthermore, unlike the method of Collins and Borysenko (1984), this procedure does not result in a significant dissociation of calmodulin during HAP chromatography and consequent low recovery of the complex from the column. This method yielded 110K-CM typically containing a molar ratio of 2 to 3 calmodulins per 110K subunit as determined by densitometry of SDS gels using purified calmodulin as a stan-

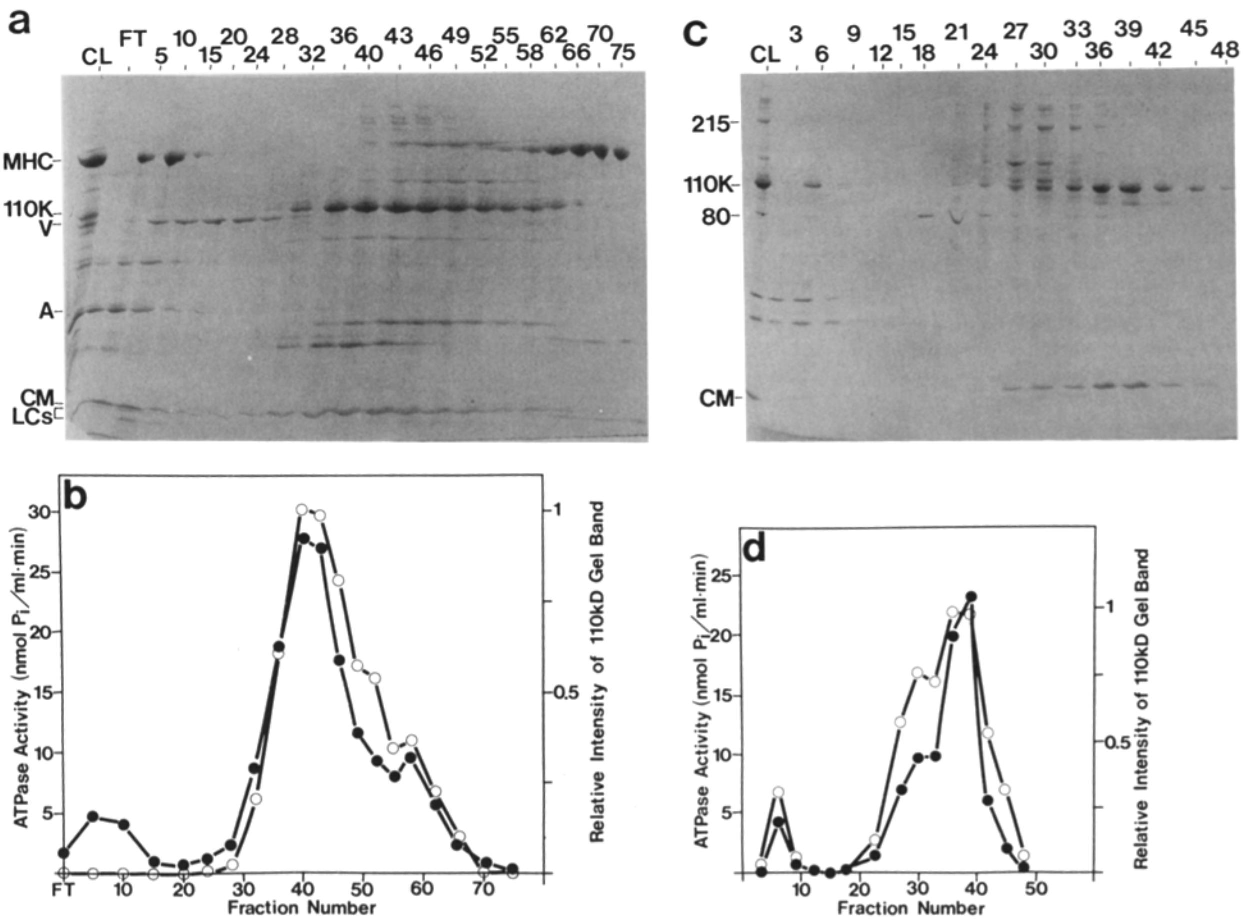


Figure 1. Purification of 110K-CM by hydroxylapatite and ion-exchange chromatography. (*a*) SDS-PAGE of HAP column fractions. *CL*, column load; proteins solubilized by treatment of brush borders with 5 mM ATP in solution I. *FT*, flow through; *MHC*, myosin heavy chain; *110K*, 110-kD subunit; *V*, villin; *A*, actin; *CM*, calmodulin; *LCs*, myosin light chains. (*b*) Elution profile of HAP column. CaATPase activity of HAP column fractions dialyzed into solution B (●). Relative intensity of Coomassie Blue-stained 110K band (○) measured by densitometry scans of SDS gels. (*c*) SDS-PAGE of Q-Sepharose column fractions. *CL*, pooled 110K-enriched fractions (Nos. 36-49) from HAP column. Fraction Nos. 40-47 were pooled from this preparation for subsequent analysis. (*d*) Elution profile of Q-Sepharose column. CaATPase activity and distribution of 110K in column fractions as for *b*.

dard for the amount of calmodulin present. SDS–PAGE of representative samples of the isolated complex are shown in Fig. 1 *c*, lane *42*, and Fig. 9, lanes *2*.

Measurement of the CaATPase activity of representative fractions across both column profiles shows that the peaks of enzymatic activity are coincident with those of 110K-CM (Fig. 1, *b* and *d*). Densitometry scans of the 110K band in SDS–polyacrylamide gels also show that there is good correlation between the intensity of Coomassie Blue staining and enzymatic activity (Fig. 1, *b* and *d*). Comparing the CaATPase activity of the initial ATP extract, and the 110K-enriched peaks from each of the columns, there is a sequential 1.6-fold and 2.4-fold increase in specific activity.

110K-CM can also be specifically fractionated from contaminating polypeptides by taking advantage of its binding affinity for F-actin. Addition of F-actin to the pooled HAP peak fractions and subsequent centrifugation at 100,000 *g* exclusively pellets the 110K-CM along with actin, leaving all other proteins in the supernatant (Fig. 2). Like 110K-CM, most of the enzyme activity is recovered in the pellet (Table I). However, this method was not used as a routine preparative step for purification of 110K-CM because in our hands, after pelleting, only ∼50% of the complex could be dissociated from the actin by ATP (see also Collins and Borysenko, 1984). In addition, subsequent attempts to purify the 110K-CM from this ATP-dissociated supernatant resulted either in significant losses of complex (Collins and Borysenko, 1984) or persistent contamination with trace amounts of actin. These difficulties led us to use ion-exchange (Q-Sepharose) chromatography as our second purification step. Nevertheless, these results do demonstrate that ATP-hydrolyzing activity copurifies with 110K-CM through three different procedures which enrich for the complex based on its biological activity as well as physical properties.

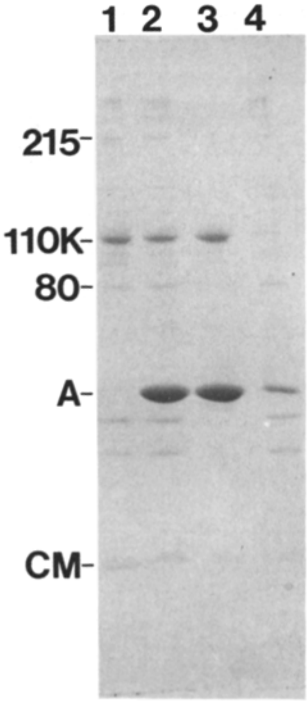


Figure 2. Fractionation of 110K-CM from HAP pooled peak fractions by actin cosedimentation. 110K-enriched fractions devoid of brush border myosin (e.g., fraction Nos. 36–49 in Fig. 1 *a*; *CL* in Fig. 1 *c*) dialyzed into solution B (lane *1*). Added prepolymerized F-actin to 0.1 mg/ml and KCl to 0.3 M (lane *2*). Pellet and supernatant fraction (lanes *3* and *4*, respectively) after centrifugation at 100,000 *g*, 60 min. Relative fractionation of ATPase activity is given in Table I.

Table I. Relative Enzymatic Activity of Fractions Separated by Cosedimentation with F-actin

Assay conditions	HAP peak	HAP peak + actin	Pellet	Supernate + actin*	Supernate
	%	%	%	%	%
MgATPase	51	100	90	15	10
CaATPase	115	100	82	18	14

Relative MgATPase and CaATPase activities of fractionation shown in Fig. 2. Compared activity to that of the pooled HAP peak fractions in the presence of actin (100% = 0.078 µmol P_i/mg per min for MgATPase and 0.375 µmol P_i/mg per min for CaATPase). For assay conditions, see Materials and Methods.

* F-actin was added to the supernatant to equivalent concentrations as the pellet.

Enzymatic Activity of 110K-CM

The ATPase activity of purified 110K-CM is dependent on the divalent cation conditions used (Table II). The highest activities are observed in solutions containing Ca^{++} or K^+ and EDTA. 110K-CM exhibits significantly less activity in the presence of Mg^{++} (Table II). This inhibitory effect of Mg^{++} on the enzymatic activity of the complex is seen most dramatically by the addition of increasing concentrations of Mg^{++} to the CaATPase assay conditions (Fig. 3). While the EDTA-ATPase activity of 110K-CM is maximal in the presence of elevated K^+ and inhibited by Na^+, the Ca and Mg ATPases are much less sensitive to the monovalent cation conditions (Table II). These results are in close agreement with the specific activities reported by Howe and Mooseker (1983) for MgATPase and by Collins and Borysenko (1984) for EDTA-ATPase, CaATPase, and MgATPase. Importantly, however, 110K-CM purified by our procedures exhibits greater MgATPase activity in the presence than in the absence of F-actin. This actin activation is a function of actin concentration (Fig. 4). Over the entire range of 110K-CM:actin ratios tested, the MgATPase activity of 110K-CM exhibits a direct dependence on actin concentration (Fig. 4) with a maximum activation of 2.5–3.5-fold at the highest actin concentration tested.

110K is the principal calmodulin-binding protein of the intestinal microvillus (Glenney and Weber, 1980; Howe et al., 1982). Unlike CM's association with most CM-regulated proteins (Klee and Vanaman, 1982), the binding of at least some of the CM to 110K is apparently Ca^{++}-independent (Glenney and Weber, 1980; Howe and Mooseker, 1983). Nevertheless, 110K-associated CM may retain a binding

Table II. Enzyme Activities of 110K-CM

Assay conditions	Average Specific Activity at 35°C (µmol P_i/mg per min)		
	K-EDTA	Ca	Mg
0.5 M KCl	0.242 (±0.073)	0.299 (±0.123)	0.056*
0.5 M NaCl	0.026 (±0.010)	0.245 (±0.097)	0.058*
75 mM KCl	0.159 (±0.079)	0.399 (±0.143)	0.053*
			0.039 (±0.010)

Effect of monovalent and divalent cations on ATPase activity of purified 110K-CM. Except as footnoted below, values reported are the average specific activities at 35°C and standard deviations for several preparations of 110K-CM (*n* = 3 for K-EDTA and CaATPases; *n* = 8 for Mg ATPase).

* Results from a single experiment.

For specific solution conditions, see Materials and Methods.

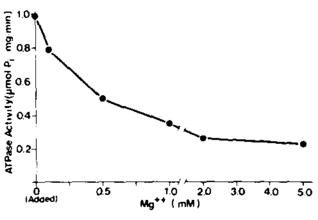


Figure 3. Relative effect of divalent cations on enzymatic activity of 110K-CM. Increasing concentrations of Mg^{++} were added to the standard solution conditions for the CaATPase assay (see Materials and Methods). Concentration of 110K-CM in this assay was 38 μg/ml.

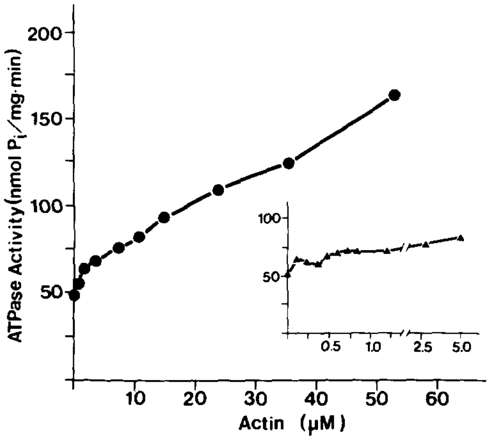


Figure 4. Effect of actin concentration on activation of MgATPase activity of 110K-CM. MgATPase activity was assayed under standard conditions (see Materials and Methods) with varying concentrations of F-actin. The level of ATPase of the actin at each concentration was assayed and its contribution was subtracted from the corresponding actin-activated activity for 110K. Concentration of 110K-CM in this representative experiment was 40 μg/ml. (*Inset*) Effect of actin concentrations from 0.1 to 5 μM on MgATPase activity. A molar excess of phalloidin was added to stabilize the actin as filaments. Concentration of 110K-CM was 25 μg/ml.

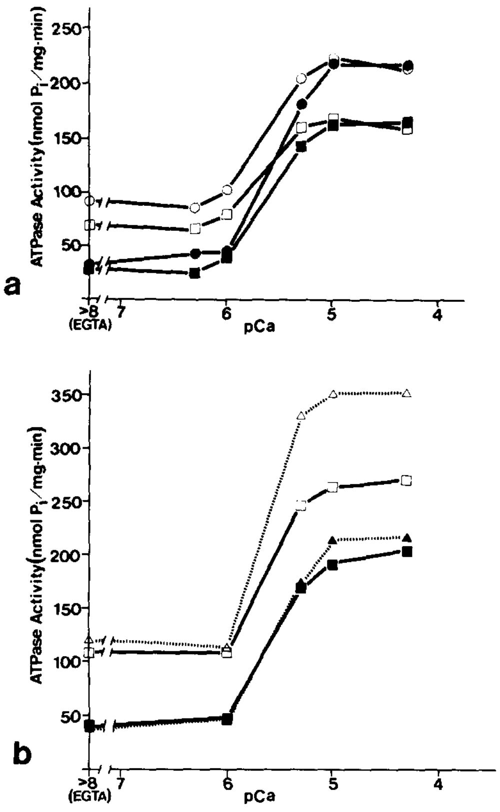


Figure 5. Effects of Ca^{++} and calmodulin on MgATPase activity. (*a*) Effect of Ca^{++} on MgATPase activity of 110K-CM. Two different preparations of 110K-CM (● and ○, 42 μg/ml; ■ and □, 72 μg/ml) were assayed for MgATPase activity in the absence (●, ■) or presence (○, □) of 0.9 mg/ml F-actin. This range of free Ca^{++} concentrations was generated using Ca-EGTA buffers (5 mM concentration of EGTA; see Materials and Methods). (*b*) Effects of exogenous CM on MgATPase activity. Actin-activated MgATPase activity was compared for 110K-CM with (*dashed lines*) and without (*solid lines*) exogenous CM (75 μg/ml) at the same free Ca^{++} concentrations shown in Fig. 5 *a*. Activity in the absence (■, ▲) and presence (□, △) of 0.9 mg/ml F-actin is reported. Concentration of 110K-CM for this experiment was 42 μg/ml.

affinity for Ca^{++} and consequently changes in Ca^{++} concentration might be expected to affect the properties of the 110K-CM complex. We examined the effect of a range of free Ca^{++} concentration from <1 nM to 50 μM on the MgATPase activity of isolated 110K-CM. Ca^{++} concentrations >5–10 μM resulted in an increase in the specific activity of 110K-CM and often also in the loss of significant actin activation (Fig. 5 *a*). Variability in the amount of associated calmodulin in the isolated complex (2–3 mol CM/mol 110K) may account for the slight variability observed from preparation to preparation in the extent of actin activation at these concentrations of free Ca^{++}. In fact, although the addition of exogenous CM to the purified 110K-CM complex (Fig. 5 *b*) had little effect on its specific activity or percent actin activation, it did maintain the actin activation to higher concentrations of Ca^{++}. The MgATPase activity of the isolated complex was not significantly affected by the addition of the CM antagonist trifluoperazine in the presence or absence of calcium (results not shown).

Differential sensitivity to the inhibitor sodium orthovanadate is one diagnostic property distinguishing various ATPases. A number of membrane-associated transport enzymes (Niggli et al., 1981; Wang et al., 1979; Cantley et al., 1977) are inhibited by concentrations of orthovanadate from 0.1 to 20 μM; whereas, much higher concentrations are required to inhibit skeletal muscle myosin (Gibbons et al., 1978; Goodno, 1979). Given 110K-CM's morphological association with the microvillus membrane and its proposed relatedness to myosin (Collins and Borysenko, 1984), we assayed the effect of orthovanadate on its enzymatic activity. Both the MgATPase and CaATPase activities of 110K-CM exhibit low sensitivity to vanadate; concentrations of 250 μM are required to obtain 50% inhibition.

Although ATP was used as the substrate for all of the experiments described above, all nucleoside triphosphates tested were hydrolyzed by 110K-CM either under conditions for CaATPase (Fig. 6 *a*) or for MgATPase (±actin) activity (Fig. 6 *b*). This is consistent with the dissociation of 110K-CM from the microvillus by all nucleoside triphosphates (NTPs). The relative extraction efficiency from the brush border was ATP > UTP, CTP > GTP, ITP (data not shown); similar results have been reported by Verner and Bretscher (1985) for solubilization of 110K from demembranated microvilli by various NTPs. The complex does not appear,

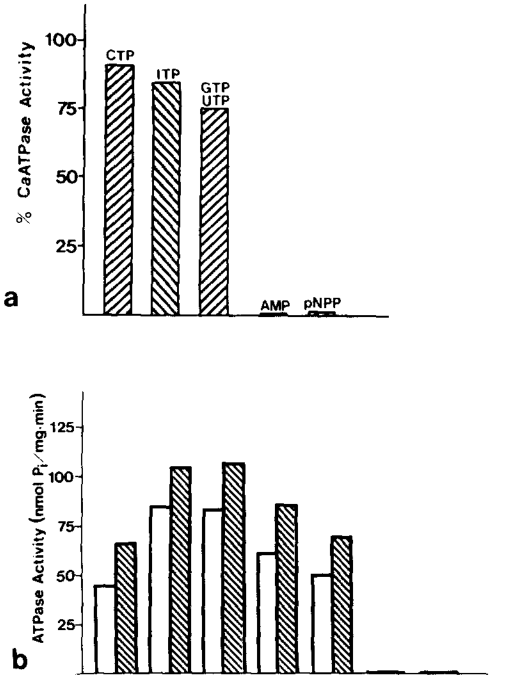


Figure 6. Substrate specificity of ATPase activities. (*a*) Substrate specificity of CaNTPase activity. Enzymatic activity is reported relative to CaATPase (100%; 0.35 μmol P$_i$/mg per min). (*b*) Substrate specificity of MgNTPase activity. (*Open bars*) Activity in absence of actin; (*striped bars*) activity in presence of 0.3 mg/ml actin.

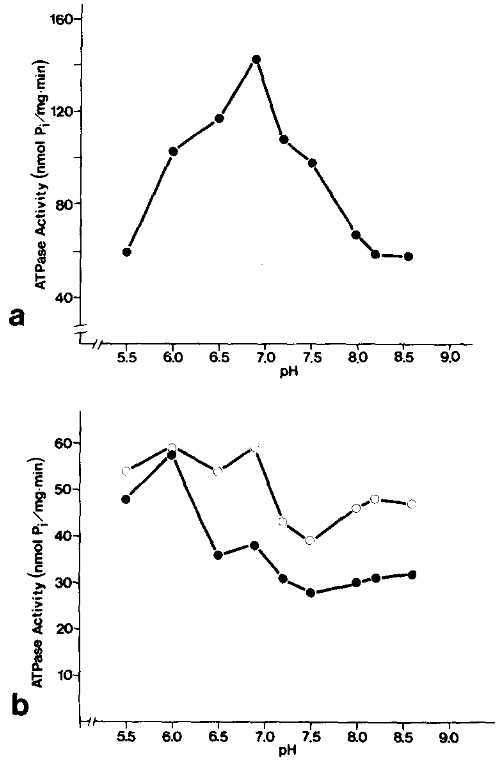


Figure 7. Effect of pH on ATPase activities. (*a*) Effect of pH on CaATPase activity of 110K-CM. (*b*) Effect of pH on MgATPase activity of 110K-CM in the absence (●) or presence (○) of 0.3 mg/ml F-actin.

however, to be a general phosphatase as neither *p*-nitrophenyl phosphate nor AMP are substrates for the enzyme (Fig. 6).

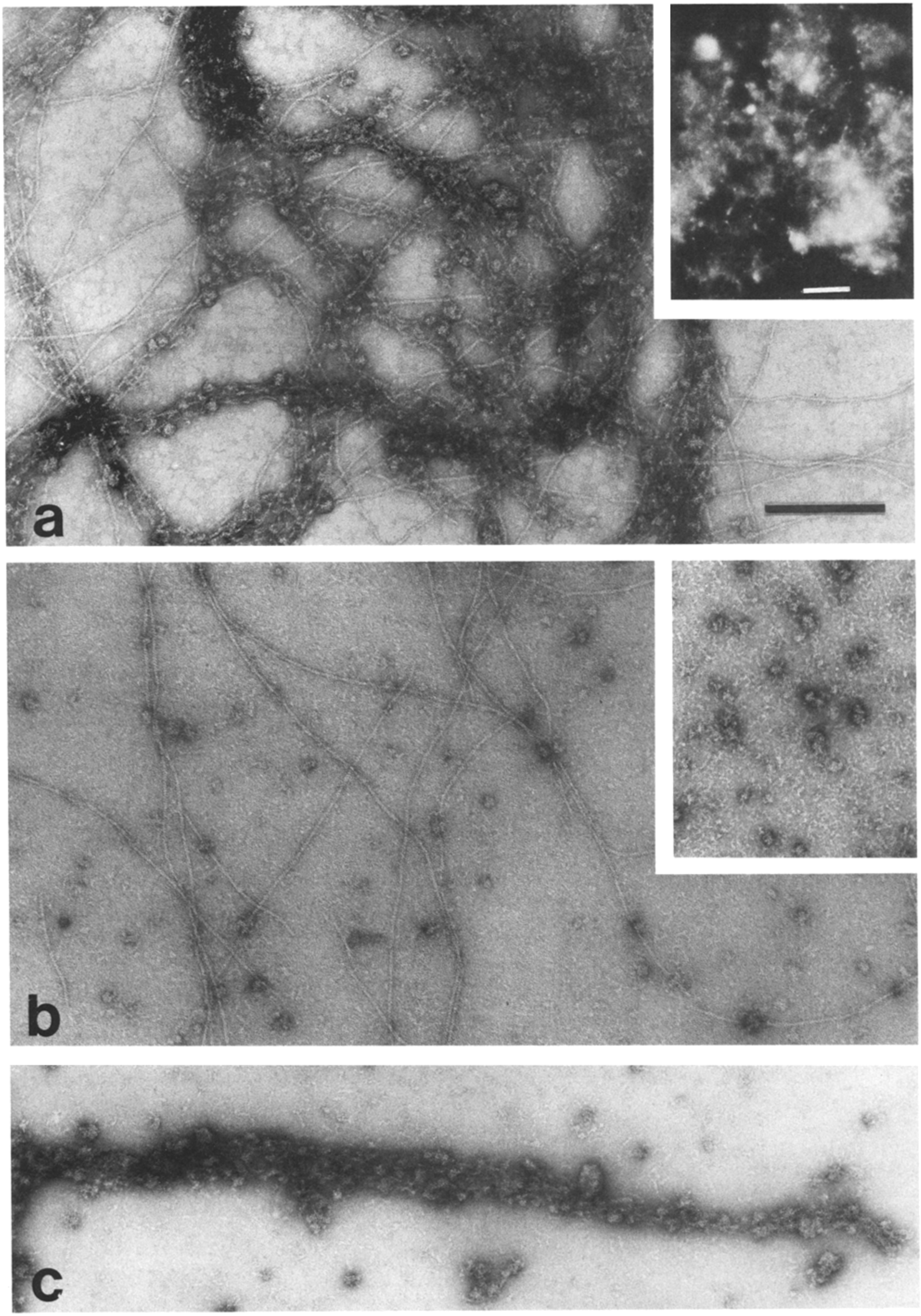
110K-CM exhibits a peak of enzymatic activity over a pH range of 6.0 to 7.5 in the presence of Ca^{++} (Fig. 7 *a*) and optimal actin activation of MgATPase from pH 6.5 to 8.5 (Fig. 7 *b*). These relatively neutral pH optima also argue against contamination with any acid or alkaline phosphatase.

ATP-dependent Cross-linking of F-actin by 110K-CM

Electron micrographs of mixtures of F-actin and partially purified 110K-CM showed loosely organized aggregates of filaments as visualized by negative stain (Howe and Mooseker, 1983). Although this apparent cross-linking of actin filaments was ATP dependent, because the preparation contained low concentrations of a 130-kD polypeptide in addition to the 110K-CM, the cross-linking activity could not be definitely attributed to 110K-CM or to 110K-CM alone. Consequently, we reexamined the interaction of F-actin with the 110K-CM complex fractionated by our procedure away from contaminating polypeptides, detectable by Coomassie Blue staining. Addition of F-actin to purified 110K-CM results in the virtually instantaneous appearance of macroscopic protein aggregates which can be easily visualized in darkfield light microscopy (Fig. 8 *a*, *inset*) or in negatively stained electron microscope preparations as randomly ordered and highly cross-linked actin networks (Fig. 8 *a*). 110K-CM can be seen in these cross-linked meshworks as globular structures associated laterally with the actin filaments. There are frequently regions with many associated 110K-CM complexes surrounded by stretches of bare actin filaments. Adding ATP results in an equally rapid disruption of these arrays as assayed by light (not shown) or electron (Fig. 8 *b*) microscopy. The 110K-CM complex is completely dissociated from the actin filaments under these conditions (Fig. 8 *b*) and is morphologically indistinguishable from the 110K-CM controls (Fig. 8 *b*, *inset*).

This ATP-dependent cross-linking of actin was also assayed by centrifugation at low speeds, conditions where most actin filaments remain in the supernatant. In the absence of ATP, 110K-CM cosediments with F-actin, and greatly increases the amount of pelletable actin (Fig. 9, compare lanes

Figure 8. ATP-dependent actin filament cross-linking by 110K-CM. (*a*) Electron micrograph of negatively stained actin-110K-CM network in solution C. Bar, 0.2 μm. (*Inset*) A comparable preparation as visualized in dark-field light microscopy. Bar, 10 μm. (*b*) Addition of 5 mM ATP disassembles the cross-linked array of actin filaments. The dissociated 110K-CM is morphologically identical to preparations of the complex in the absence of actin (*inset*). In these samples, a few spheroid aggregates are seen in a field of smaller molecules. Actin concentration, 0.05 mg/ml; 110K-CM, 0.05 mg/ml. (*c*) Negatively stained preparation of 110K-CM and F-actin in solution C at a weight ratio of 50:1. At this elevated ratio, the actin is apparently saturated with 110K-CM and is primarily cross-linked into bundles of two and three filaments. Consistent with the cosedimentation results at this ratio (Fig. 10, lane *5*), unbound 110K-CM is visible in the background. Actin concentration, 1 μg/ml; 110K-CM, 50 μg/ml.


a
b
c

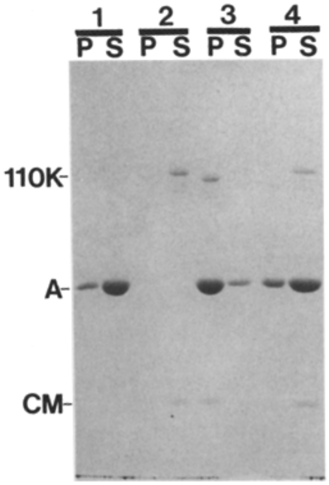


Figure 9. Low speed cosedimentation of actin-110K-CM networks. Macroscopic 110K-CM-actin meshworks similar to those visualized in Fig. 8 *a* are pelleted (*P*) (lanes *3*) at speeds at which most F-actin alone (lanes *1*) remains in the supernatant (*S*). 110K-CM alone is also soluble under these conditions (lanes *2*). This filament cross-linking is ATP dependent (lanes *4* were incubated in the presence of 5 mM ATP) and reversible (not shown but identical to lanes *4*). Bands below 110K are proteolytic fragments of 110K as determined by immunoblot analysis with affinity-purified antibody to 110K (not shown). Actin concentration was 0.2 mg/ml.

3 to lanes *1*). This effect is ATP dependent and reversible. Adding ATP before or after addition of F-actin (Fig. 9, lanes *4*) results in the nonsedimentation of 110K-CM and most of the actin.

Although there was no apparent periodicity to the binding of 110K-CM and actin in these cross-linked arrays, the molar concentration of actin used far exceeded that of 110K-CM. Since the highly ordered decoration of F-actin by myosin or its proteolytic fragments (e.g., subfragment-1, S_1) (Huxley, 1963; Pollard, 1981) is best visualized at saturating ratios of myosin head to actin, we also examined the structural organization of 110K-CM associated with actin filaments at ratios greater than that determined by cosedimentation (Fig. 10) to be approximately saturating (mass ratio of 20:1; based on a molecular mass of 170 kD for 110K-CM, this corresponds to a molar ratio of $\sim$5:1). Under these conditions, the actin filaments are dramatically coated with 110K-CM (Fig. 8 *c*); however, there is still no obvious periodicity of 110K-CM binding similar to S_1 decoration or to the helical arrangement of the lateral bridge in the microvillus (Matusadaira and Burgess, 1982*a*). In contrast to the meshworks observed at high concentrations of actin (Fig. 8 *a*), at this 110K-CM:actin ratio, the actin is primarily observed as bundles of two to three filaments.

Visualization of 110K-CM Molecules by Rotary Shadowing

Although negative stain proved to be a useful technique in the examination of properties of 110K-CM (see above), this method of specimen preparation provided little information on the structure of the complex itself (Fig. 8 *b*, *inset*). Deep-etch replicas of these same samples (done by Dr. John Heuser, Washington University) revealed that the complex is primarily in the form of a single-headed molecule with a more globular head region and short tail (Fig. 11, *top panel* and rows *a–c*). Some of these "tadpole-shaped" molecules are associated in clusters of 3–7 (Fig. 11, *top panel* and row *f*); in all cases, the individual molecules in these multimeric units are oriented with their tail regions associated with a blob of additional material and their thicker head domains extending away from the aggregate. These images are quite consistent with the micrographs of negatively stained preparations where larger spheroid aggregates are seen in a field of many smaller molecules (Fig. 8 *b*, *inset*).

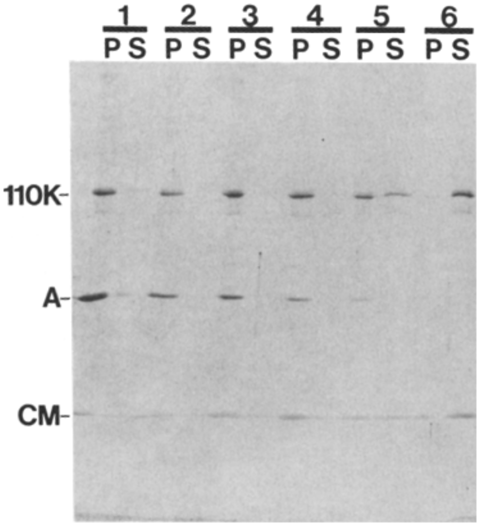
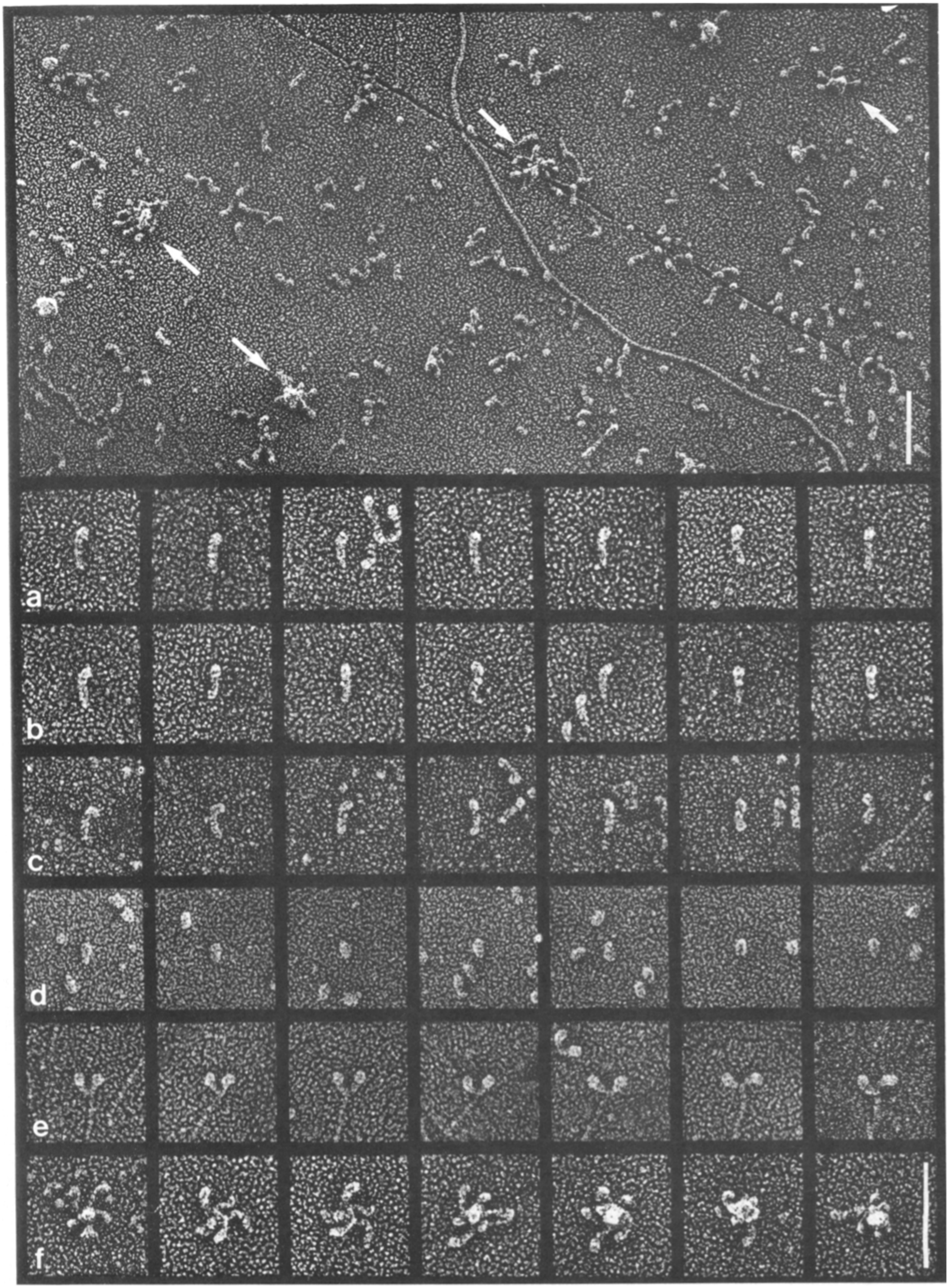


Figure 10. Actin filament binding at higher ratios of 110K-CM. Determination of apparent saturating ratios of 110K-CM to actin by cosedimentation. A fixed concentration of 110K-CM (50 µg/ml) was incubated with various concentrations of F-actin (25, 10.5, 2.5, 1, 0 µg/ml in lanes *1–6*, respectively) before centrifugation. The extent of actin binding was assessed by fractionation into pellet (*P*) and supernatant (*S*) fractions. At low 110K-CM/actin ratios, all the 110K-CM is pelleted with the filaments (lanes *1–3*); however, as ratios are increased (>20:1 by mass; lanes *4* and *5*), only a fraction of the 110K-CM is pelletable.

Discussion

While there is signficant variability in the molecular weight, composition, and mechanisms of regulation of myosins isolated from skeletal muscle, smooth muscle, and nonmuscle sources, all myosins exhibit the following diagnostic enzymatic characteristics (Pollard, 1982): (*a*) high ATPase activ-

Figure 11. Comparison of deep-etch replicas of 110K-CM complex and proteolytic fragments of skeletal muscle myosin. Survey view (*top panel*) and rows *a* and *b* display purified 110K-CM adsorbed to mica in solution C, then freeze-dried and rotary replicated with platinum. Row *c* illustrates the same preparation after a 15-min fixation in 0.4% glutaraldehyde; no significant differences in size or shape are seen. For comparison, rows *d* and *e* illustrate myosin subfragment-1 and heavy meromyosin, respectively. The two heads of such HMM preparations measure $\sim$20 nm in length, versus $\sim$15 nm for myosin S_1 and $\sim$30 nm for the 110K-CM. All three proteins measure $\sim$10 nm diameter at their widest portions. Row *f* displays several examples of the aggregates found in all our 110K-CM preparations (*top panel*, *arrows*). These appear to be composed of 5 ± 2 of the above 110K-CM species all emanating from a central 10–20-nm granule of unidentified composition. The long filament crossing the field in the survey view is a keratin contaminant. This plate was kindly prepared for us by Dr. John Heuser of Washington University, St. Louis, MO. Bars, 50 nm.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ity in the presence of potassium and EDTA and/or in the presence of Ca^{++}; (*b*) relatively low activity in the presence of Mg^{++}; and (*c*) enhancement of MgATPase activity by F-actin.

The observations of Collins and Borysenko (1984) first raised the exciting possibility that the microvillus protein complex, 110K-CM, might be a myosin-like enzyme. The data reported here confirm their results for the K-EDTA, Ca, and Mg ATPase activities (Table II, Fig. 3) but also provide additional information regarding the pH optima (Fig. 7), substrate specificity (Fig. 6), and effect of Ca^{++} on 110K-CM's enzymatic activities (Fig. 5). Most importantly these data also show that the 110K-CM complex exhibits the third characteristic of a myosin-like enzyme: it is an actin-activated MgATPase under physiological conditions. Like myosin (Eisenberg and Moos, 1968), the level of activation is a function of actin concentration (Fig. 4). However, this activation does not exhibit the simple hyperbolic kinetics typical of most myosins. There is also no indication of a triphasic activation response as is observed for the globular *Acanthamoeba* myosin I (Pollard and Korn, 1973*b*; Albanesi et al., 1985*b*) even at the highest 110K-CM:actin ratios tested (actin concentrations from 0.1 to 5 μM) (Fig. 4, *inset*).

Compared to most myosins, the actin activation of 110K-CM is relatively low (at most 2.5–3.5-fold). While this may be the maximal activation of the enzyme, it is also possible that the MgATPase activity is regulated by an additional factor fractionated away from the complex during its isolation. For example, many myosins are regulated by phosphorylation either of their light chains (for review see Adelstein and Eisenberg, 1980; Chacko et al., 1985), and/or their heavy chains (Maruta and Korn, 1977; Cote et al., 1985; Collins and Korn, 1980; Kuczmarski and Spudich, 1980; Peltz et al., 1981). In situ, the 110K subunit is a phosphorylated protein (Keller and Mooseker, 1982) and although, as yet not observed, brush border CM may also be phosphorylated in vivo (by analogy with the convention for myosin, 110K would be the "heavy chain" and CM the "light chain" of the complex). It is possible that the absence of specific regulatory kinase(s) or phosphatase(s) from the assay mixture accounts for the low activation observed in vitro. These experiments have also not ruled out the possibility that binding of 110K-CM to another polypeptide, which may or may not be dissociated from the brush border by ATP, (e.g., an integral membrane protein of the microvillus) stimulates the enzymatic activity of the complex.

The MgATPase activity of many myosins is regulated directly by Ca^{++} or indirectly by Ca^{++} and calmodulin. Since the intestinal epithelium is the body's primary site of vitamin D–dependent calcium absorption (for review see Bikle et al., 1981; Bronner et al., 1986), there may be significant fluxes in transepithelial calcium transport and in the consequent amount of calcium bound to the complex. In vitro, the addition of 5–10 μM Ca^{++}, concentrations sufficient to saturate the binding affinities of CM (Klee and Vanaman, 1982), stimulates the specific activity of the 110K-CM (Fig. 5 *a*). However, this calcium-dependent potentiation of enzymatic activity is often concommitant with a decrease in the extent of actin activation (Fig. 5 *a*). While this may reflect a physiologically significant uncoupling of the enzymatic activity from the cytoskeleton or a calcium-regulated change in function, the extent of actin activation may also be affected by the amount of CM associated with the complex. Calmodulin binding might be expected to change the conformation, stability, and/or solubility of the 110K-CM complex. Although the complex has a ratio of 2-3 mol of CM per mol of 110K as isolated, it is purified under conditions (i.e., in the presence of EGTA) which would tend to dissociate CM from any Ca^{++}-dependent binding sites. Preliminary results from Swanljung-Collins et al. (1986) suggest that there may be as many as four CM-binding sites per 110K subunit. In vitro, the addition of exogenous CM maintains the actin activation of 110K-CM to higher concentrations of calcium (Fig. 5 *b*). Under these conditions, the addition of actin and 5–10 μM Ca^{++} stimulates the MgATPase activity of the enzyme sevenfold. This effect may be mediated by Ca^{++} binding to CM and/or to high affinity Ca^{++}-binding sites on the 110K subunit itself.

110K-CM also shares two other important properties with muscle and nonmuscle myosins: binding to F-actin to form ATP-dissociable complexes and cross-linking of actin filaments (Pollard, 1981). The ATP-dependent actin-binding activity of 110K-CM was first suggested by the dissociation of the lateral bridge from demembranated microvillus cores by ATP (Matsudaira and Burgess, 1979). Direct evidence for the actin-binding activity of the complex was provided by cosedimentation of 110K-CM with F-actin (Howe and Mooseker, 1983; Collins and Borysenko, 1984; Verner and Bretscher, 1985). These results are consistent with the solubilization of 110K-CM from the brush border cytoskeleton by ATP and also with its structural organization in the microvillus. To date, 110K-CM and myosin are the only known ATP-dependent actin-binding proteins.

Considering 110K-CM's topographic organization as a lateral link between the microvillus core and the membrane, its actin cross-linking properties are somewhat puzzling. Clearly, filament cross-linking requires at least two actin-binding sites per molecule. While the observed cross-linking activity of 110K-CM may be accounted for by the small percent of molecules associated into multimeric units (Fig. 11, *top panel* and row *f*), the monomeric 110K-CM (Fig. 11, *top panel* and rows *a* and *b*) may also be a bivalent actin-binding moiety. There is evidence for cross-linking of actin filaments by the globular *Acanthamoeba* myosin I (Pollard and Korn, 1973*b*; Fujisaki et al., 1985) and by the S1 proteolytic fragment of skeletal muscle myosin (Ando and Scales, 1985). The cross-linking capability of these other single-headed and apparently tailless (Albanesi et al., 1985*a*; Lynch et al., 1986) polypeptides cannot be attributed either to the assembly into bipolar filaments or the double-headed structure thought to account for the cross-linking of F-actin by more typical myosins. In the case of myosin IA, two discrete actin-binding domains have been localized to distinct regions of the molecule (Lynch et al., 1986).

The nature of the association of 110K-CM into the observed multimers is an unresolved question. Although this may represent artifactual aggregation, their relatively uniform appearance and the apparent polarity of the monomers (heads pointed away) (Fig. 11, row *f*) within these aggregates suggest that the interaction may be specific. For example, the material connecting the tail regions into clusters may be a remnant of the membrane-linkage component of this microvillus cross-bridge (e.g., associated phospholipid or a soluble fragment of associated integral membrane proteins

[Coudrier et al., 1983]). These aggregates may also explain the high 110K-CM:actin molar ratio at apparent saturation for binding (Fig. 8 *c*). Although most of the isolated 110K-CM is present as single molecules (Fig. 8 *b*, *inset*; Figure 11, *top panels* and rows *a*–*c*), these may associate into higher order aggregates in regions of locally high concentration such as the surface of actin filaments. Electron micrographs of these preparations of 110K-CM and F-actin are consistent with most of the bound 110K-CM being multimeric (Fig. 8 *c*).

Although the physiological significance of the ATPase activity of 110K-CM has yet to be determined, the similarity of the enzymatic properties, binding activities, and vanadate sensitivities of 110K-CM and myosin suggests the functional relatedness of the two proteins. The amoeboid myosin I's provide precedents for such a low molecular mass, globular (Pollard and Korn, 1973*a*; Cote et al., 1985), and membrane-associated (Gadasi and Korn, 1980; Adams and Pollard, 1986) myosin. In addition, preliminary results (Carboni, J., T. Shibayama, K. Conzelman, and M. Mooseker, unpublished observations) using an affinity-purified polyclonal antibody generated against avian 110K suggest that the 110K subunit shares antigenic determinant(s) with the heavy chain of smooth and skeletal muscle myosin. At least some of the common epitopes have been localized to the actin-binding and enzymatic head domain (S_1 proteolytic fragment) of skeletal muscle myosin. While all of these data are consistent with the myosin-like nature of 110K-CM, the final proof of their functional relatedness will be a demonstration that the complex can transduce chemical energy to mechanical work. Preliminary experiments (Mooseker, M., K. Conzelman, and M. Sheetz, unpublished observations) using the in vitro *Nitella* bead movement assay (Sheetz and Spudich, 1983; Sheetz et al., 1984) suggest that 110K-CM is in fact a mechanoenzyme, supporting directional, ATP-dependent motility along actin filaments. However, further experiments, currently in progress in our laboratory, are needed to show conclusively that 110K-CM is the "motor" responsible for this movement as a preparation of microvillus membrane vesicles, highly enriched for 110K-CM, was used for these studies rather than the purified complex.

One can envision a variety of roles a motile 110K-CM could play in the brush border (e.g., moving integral membrane proteins within the plane of the membrane, directionally transporting vesicles between the cell body and the microvillus membrane, moving the microvillus core bundle toward the terminal web, or rotating it relative to the membrane). Determination of the physiological significance of this myosin-like and membrane-associated protein complex may be important in integrating the structural aspects of the brush border cytoskeleton with its functional role in the absorptive epithelium.

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