Preparation and Characterization of Heavy Meromyosin and Subfragment 1 from Vertebrate Cytoplasmic Myosins†

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ABSTRACT: The soluble fragments of myosin, heavy meromyosin (HMM), and subfragment 1 (S-1) have been instrumental in elucidating the kinetic mechanisms of the actin-activated MgATPase activity of both skeletal and smooth muscle myosin. To date, relatively little has been published on these fragments from vertebrate cytoplasmic myosins. We now describe the preparation and steady-state kinetic characterization of S-1 and HMM from human platelet and avian intestinal epithelial brush border myosin. The HMM prepared from each of these tissues was similar both in their SDS–polyacrylamide gel pattern and in their steady-state kinetic properties. The V_{max} of the actin-activated MgATPase activity varied between 0.8 and 2.5 s^{-1}, and the K_{ATPase} (the apparent dissociation constant derived from a double-reciprocal plot of the MgATPase activity) was about 1–2 μM. This low value for the apparent dissociation constant was similar to the dissociation constant of HMM for actin directly measured under similar conditions and is about 40 times lower than that determined with avian smooth muscle HMM. The K_{ATPase} of the cytoplasmic HMM was only slightly increased when the ionic strength was raised from 12 to 112 mM.

Myosin is the enzyme which, along with actin, converts the chemical energy present in ATP to a mechanical force during muscle contraction (Adelstein & Eisenberg, 1980; Eisenberg & Hill, 1985). In nonmuscle cells, myosin is thought to perform a similar contractile function (Sellers & Adelstein, 1987). The myosin molecule is composed of six polypeptide chains: There are two heavy chains (200 000 Da) and two pairs of light chains (termed regulatory and essential). In most vertebrate cytoplasmic myosins, the regulatory light chain has a molecular mass of 20 000 Da while that of the essential light chain is 17 000 Da (Sellers & Adelstein, 1987). The carboxy-terminal portion of the two heavy chains forms a coiled-coil α-helical region of about 150 nm in length which is termed the rod. Beyond this point, the two heavy chains diverge to form two more globular regions termed "heads". Each head has an actin binding site and an ATP hydrolysis site and is associated with one of each class of light chains.

At low ionic strength, myosin associates to form large filaments. Filament assembly of smooth and nonmuscle myosins is regulated at least in vitro by phosphorylation of the 20 000-Da light chain (Suzuki et al., 1978; Scholey et al., 1980). Phosphorylation of the 20 000-Da light chain also leads to an activation of the actin-activated MgATPase activity of the myosin (Sobieszek & Small, 1977; Chacko et al., 1977; Ikebe et al., 1978; Sherry et al., 1978; Adelstein & Conti, 1975; Sellers et al., 1981). In the case of turkey gizzard smooth muscle myosin, this activation is the result of a large increase in the rate constant for a single kinetic step, the release of P_i from an acto–myosin·ADP·P_i complex (Sellers, 1985).

The assembly of myosin into filaments makes it difficult to study the interactions of individual myosin molecules with actin by most conventional kinetic techniques including spectroscopy and analytical ultracentrifugation. Thus, most detailed kinetic studies of myosin have been performed using soluble, enzymatically active proteolytic fragments of myosin. Two such fragments have been prepared and characterized from skeletal and smooth muscle myosin (Adelstein & Eisenberg, 1980). One of these, termed heavy meromyosin (HMM),[1] is a soluble two-headed fragment which contains a portion of the coiled-coil α-helical rod region of the parent myosin molecule. HMM prepared from turkey gizzard myosin by chymotryptic digestion contains both types of light chains and has a MgATPase activity which is regulated by phosphorylation (Sellers et al., 1981; Sellers, 1985). The other fragment is a single-headed molecule termed subfragment 1 (S-1) whose MgATPase activity is not regulated by phosphorylation of the 20 000-Da light chain (Seidel, 1980; Ikebe & Hartshorne, 1985; Greene et al., 1983).

To date, there has been almost no characterization of the corresponding fragments from vertebrate cytoplasmic myosins. We have now studied the proteolytic digestion of human platelet and avian intestinal epithelial brush border myosins in order to prepare HMM and S-1. We have prepared each of these fragments in sufficient quantities to elucidate their steady-state kinetic properties. These preparations should contribute toward an understanding of the mechanism of the MgATPase activity of vertebrate cytoplasmic myosins. We find that in both of these myosins, there is an extremely proteolytically sensitive region located approximately 70 kDa from the amino terminus of the molecule. Digestion at this site does not result in dissociation of the cleaved polypeptide chains under nondenaturing conditions, and further digestion results in cleavage at the HMM or S-1 sites, allowing for the isolation of these two fragments.

Interestingly, we find that the apparent binding constant of each of these fragments to actin is much larger than that reported for the corresponding fragments from turkey gizzard smooth muscle, skeletal muscle, and cardiac muscle myosin.

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[1] Abbreviations: HMM, heavy meromyosin; S-1, subfragment 1; LMM, light meromyosin; Mops, 4-morpholinepropanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DFP, diisopropyl fluorophosphate; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone.

Some of these data have been reported in preliminary form (Sellers et al., 1985; Bengur et al., 1986).

Experimental Procedures

Preparation of Proteins. Smooth muscle myosin (Sellers et al., 1981), heavy meromyosin (Sellers et al., 1981), and S-1 (Greene et al., 1983) were prepared from turkey gizzards as previously described. Myosin was prepared from fresh human platelets which had been washed 3 times in 154 mM NaCl, 5 mM EDTA, and 10 mM Tris·HCl (pH 7.4). The platelets were disrupted by homogenization in a Teflon–glass tissue grinder in 0.25% Triton X-100, 0.5 M NaCl, 50 mM Mops (pH 7.0), 5 mM EDTA, 10 mM EGTA, 5 mM ATP, 2 mM dithiothreitol, 0.1 mM PMSF, 10 mg/L pepstatin, 10 mg/L aprotinin, 10 mg/L TPCK, 10 mg/L leupeptin, 10 mg/L antipain, 5 mg/L chymostatin, 0.1 mM DFP, and 10 mg/L TLCK. The homogenate was centrifuged for 30 min at 200000*g*. The supernatant was made 30 mM in $MgSO_4$, and an additional 5 mM ATP was added. These additions were made while maintaining the pH between 6.8 and 7.0 with the addition of Tris base. Saturated ammonium sulfate was slowly added with stirring to a final saturation of 40%. The solution was centrifuged for 10 min at 12000*g*, and the supernatant was brought to 60% of saturation with ammonium sulfate and was again centrifuged for 20 min at 13000*g*. The resulting pellet was dissolved in 25 mM NaCl, 10 mM Mops (pH 7.0), 10 mM $MgCl_2$, 0.1 mM EGTA, 1 mM dithiothreitol, 0.1 mM PMSF, 10 mg/L pepstatin, 10 mg/L TPCK, 10 mg/L chymostatin, and 5 mg/L leupeptin and was dialyzed overnight. The contents of the dialysis sack were centrifuged at 40000*g* for 20 min, and the pellet was resuspended in a small amount of dialysis solution. If the crude myosin had less than 20% actin contamination as determined by SDS–polyacrylamide gel electrophoresis, it was made 0.5 M in NaCl and 10 mM in MgATP (by addition of stock solutions of 5 and 0.1 M, respectively) and applied directly to a Sepharose 4B column (2.5 × 90 cm) which was equilibrated with 0.5 M NaCl, 10 mM Mops (pH 7.0), 0.1 mM EGTA, 1 mM dithiothreitol, and 3 mM NaN_3. Prior to application of the crude myosin, 100 mL of the column buffer containing 1 mM MgATP was applied to the column. If the crude myosin showed a larger actin contamination, a second ammonium sulfate fraction was performed as described above, taking the fraction sedimenting between 45% and 60% of saturation. The resulting pellet was solubilized in column buffer and chromatographed as described above.

Turkey epithelial brush border myosin was prepared and extracted as described by Citi and Kendrick-Jones (1986). Myosin was fractionated from the extracted supernatant by ammonium sulfate as described above for the platelet and was purified by Sepharose 4B chromatography.

Subfragment 1 (S-1) was prepared from either purified platelet or brush border myosin by digestion with papain (Worthington) under the following conditions: 0.5 M NaCl, 10 mM Mops (pH 7.0), 0.1 mM EGTA, and 1 mM dithiothreitol at 25° for 12 min at a ratio of 0.2 units of papain/mg of myosin. Papain was activated according to Lowey et al. (1969). The digestion was terminated by addition of 100 mM iodoacetic acid (pH 7.3) to a final concentration of 5 mM. The digested myosin was then dialyzed against 25 mM NaCl, 10 mM Mops (pH 7.0), 10 mM $MgCl_2$, 0.1 mM EGTA, and 1 mM dithiothreitol. The solution was then centrifuged at 40000*g* for 20 min, and the supernatant, which contained S-1, was collected and stored on ice.

Heavy meromyosin was prepared from brush border or platelet myosin by chymotryptic digestion. The myosin (3 mg/mL) was first phosphorylated by incubation for 5 min at 25 °C in 25 mM NaCl, 10 mM $MgCl_2$, 10 mM Mops (pH 7.0), 0.1 mM EGTA, 0.2 mM $CaCl_2$, 1 mM ATP, 1 mM dithiothreitol, 10^{-5} M calmodulin, and 10^{-8} M myosin light chain kinase; 5 M NaCl and 0.5 M EGTA were then added to final concentrations of 0.5 M and 5 mM, respectively, and chymotrypsin (Worthington) was added to a final concentration of 0.02 mg/mL. The digestion proceeded for 10 min and was stopped by the addition of 0.2 mM DFP and 0.2 mM PMSF. The digest was dialyzed against 25 mM NaCl, 10 mM $MgCl_2$, 10 mM Mops (pH 7.0), 0.1 mM EGTA, 0.1 mM PMSF, and 1 mM dithiothreitol, and HMM was collected from the supernatant following centrifugation at 40000*g* for 20 min. It could be stored on ice for about 1 week without further degradation or significant decrease in activity. The 20-kDa light chain of the HMM was found to be greater than 90% phosphorylated by glycerol–urea gel electrophoresis. Longer digestion times resulted in production of S-1 and were thus avoided.

Turkey gizzard myosin light chain kinase (Adelstein & Klee, 1981) and bovine brain calmodulin (Klee, 1977) were purified as previously described. Rabbit skeletal muscle actin was prepared by a modification of the method of Spudich and Watt (Eisenberg & Kielley, 1974; Spudich & Watt, 1971).

Assays. The MgATPase activity of S-1 or HMM was measured at 25 °C in 10 mM imidazole (pH 7.0), 2 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP, 0.1 mM EGTA, and 1 mM dithiothreitol at actin concentrations ranging from 0 to 100 μM. The S-1 or HMM concentration was usually 5×10^{-8} M.

The binding of HMM to actin was measured in a final volume of 0.8 mL under the same conditions as described above for the MgATPase assay except that 1 mg/mL bovine serum albumin was added. The samples were mixed and centrifuged at 470000*g* for 8 min in a Beckman TL100 ultracentrifuge. The top 0.5 mL of the solution was removed and added to a tube containing 0.5 mg in a volume of 0.05 mL of rabbit skeletal muscle actin. The MgATPase of each sample was measured and compared to that of a sample which contained no actin during the binding assay.

In both types of MgATPase assays, four or more time points were taken to ensure linearity of the MgATPase activity. The method of Martin and Doty (1948), as modified by Pollard and Korn (1973), was used to determine inorganic P_i release.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out according to Laemmli (1970), in the microslab gel apparatus described by Matsudaira and Burgess (1978). Glycerol–urea–polyacrylamide gel electrophoresis was performed according to Perrie and Perry (1970).

Results

Preparation of HMM and S-1 from Vertebrate Cytoplasmic Myosin. In order to produce the soluble, enzymatically active fragments S-1 and HMM from platelets, we tried digestion with papain and chymotrypsin. Digestion of unphosphorylated platelet myosin with papain (Figure 1A) results in a complex pattern of peptides. On the basis of digestion studies of turkey gizzard smooth muscle myosin, we were able to elucidate the following sequence of cleavages (Sellers & Harvey, 1984). The most rapidly cleaved site is located approximately 75 kDa from the amino terminus, resulting in peptides with SDS–polyacrylamide mobilities of 140 and 75 kDa. The 140-kDa peptide comes from the carboxy terminus, and the 75-kDa peptide comes from the amino terminus. This cleavage site (termed site III; Sellers & Harvey, 1984) is within the S-1 domain. Cleavage at site III does not result in dissociation

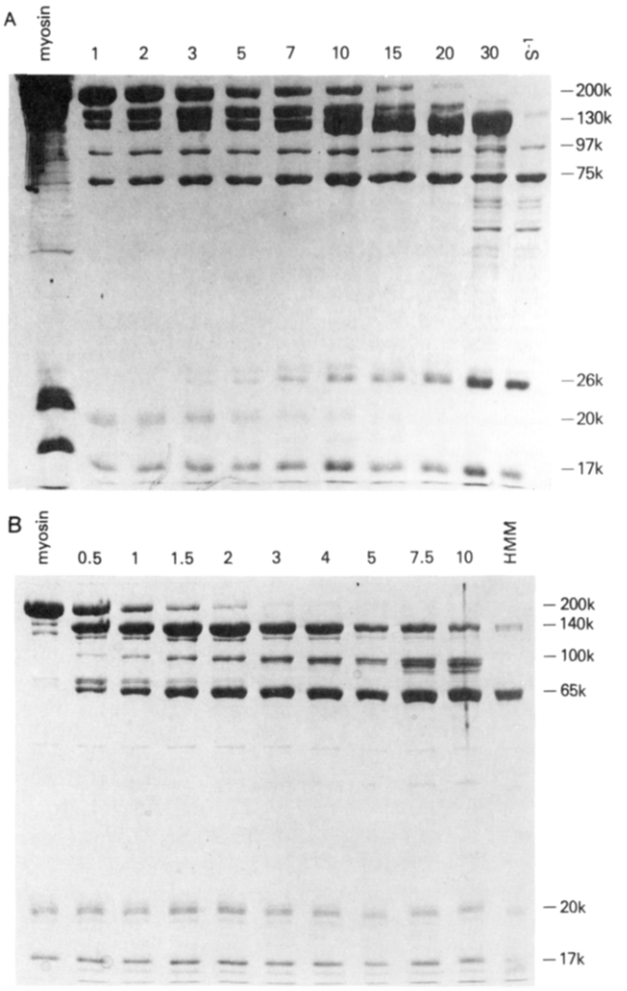

FIGURE 1: Digestion of platelet myosin by papain and chymotrypsin. (A) Platelet myosin was digested with papain as described under Experimental Procedures. Aliquots were removed at the times indicated at the top of the gels (in minutes) and made 5 mM in iodoacetic acid and then electrophoresed (5 μg per sample) except for the intact myosin which was overloaded (20 μg) on 12.5% SDS–polyacrylamide gels. The last column is the platelet S-1 preparation which was purified as described under Experimental Procedures. Note the lack of 20-kDa light chains in the digest of myosin. (B) Platelet myosin was digested with chymotrypsin as described under Experimental Procedures. Aliquots were removed at the times indicated at the top of the gels (in minutes) and made 1 mM in PMSF. The samples were then electrophoresed (5 μg per sample) on 12.5% SDS–polyacrylamide gels. Note the retention of 20-kDa light chain.

of the heavy-chain peptides under nondenaturing conditions. Cleavage also occurs at the S-1/rod junction at a slower rate. The end result of such a digestion is seen in the 30-min sample in Figure 1A where there are three predominant bands: a 130-kDa rod fragment and a pair of bands at 75 and 26 kDa representing S-1 which has been cleaved internally at site III. There is a small amount of intact S-1 present at 97 kDa. Papain digestion rapidly removes the 20-kDa light chain. S-1 is obtained by dialysis of this material against a low ionic strength buffer followed by centrifugation to remove myosin rod and undigested myosin. The S-1 thus obtained is shown in the last lane of Figure 1A.

Digestion of phosphorylated platelet myosin by chymotrypsin in the presence of ATP also gives a relatively complex SDS–polyacrylamide gel pattern since chymotrypsin also cleaves myosin rapidly at site III (Figure 1B). We found that the best way to monitor for the production of HMM was to follow the accumulation of light meromyosin (LMM) which appears as a heterogeneous collection of bands of about 100

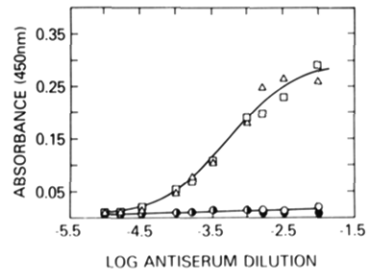

FIGURE 2: Reactivity of a monoclonal antibody prepared against turkey gizzard smooth muscle myosin toward avian intestinal smooth muscle myosin and brush border cytoplasmic myosin. (●) Actin (100 ng) or purified myosin (500 ng) from either gizzard smooth muscle (□), intestinal smooth muscle (△), or brush border epithelium (○) was dried onto a 96-well plate and washed with TBS and then blocked with TBS + 20% fetal calf serum. Monoclonal antibody TGM3 (Schneider et al., 1985) which was prepared against gizzard smooth muscle myosin was added at the indicated dilutions for 2 h. Unreacted antibody was removed by washing with TBS, then TBS + NP-40, and then TBS again. Horseradish peroxidase labeled rabbit anti-mouse IgG (1:2000 dilution) was then added for 1 h. Unreacted antibody was removed by the washing procedure described above, color was developed for 15 min using the Bio-Rad kit, and the plates were read at 450 nm. The results were the average of four individual determinations.

kDa in molecular mass (Figure 1B). Heavy meromyosin could be prepared by dialysis of the chymotryptic digest against a low ionic strength buffer which results in precipitation of LMM (M_r 100K) and undigested myosin (M_r 200K and 140K + 70K). Figure 1B (last lane) shows an SDS–polyacrylamide gel of the HMM preparation. Most of the heavy chain is cleaved internally at site III to give a pair of bands with molecular masses of 70 and 65 kDa. Some intact HMM heavy chains with a molecular weight of 140K are present. Evidence that the material produced is indeed HMM is that it is soluble at low ionic strength and that it comigrates with turkey gizzard HMM and not S-1 or intact myosin upon gel filtration (data not shown). In addition, further digestion of platelet HMM by chymotrypsin does not result in further LMM production, suggesting that the 140K band does not contain myosin rod. Phosphorylation of the 20-kDa light chain results in less cleavage of this light chain during digestion. All of the 20-kDa light chain was phosphorylated and remained so for several weeks if the preparation was stored on ice. If the digestion is carried out on unphosphorylated myosin in the absence of ATP, the light chain is cleaved much more rapidly. The yield of HMM is not very large (about 20%) since long digestion times are avoided in order to minimize loss of the 20-kDa light chain and production of S-1. If bands corresponding to S-1 (i.e., 75 kDa and 26 kDa) are observed upon SDS–polyacrylamide gel electrophoresis, the HMM was not used for kinetic analyses.

We also isolated myosin from turkey intestinal epithelial brush border in order to prepare its fragments. We used a smooth muscle specific monoclonal antibody (Schneider et al., 1985) in order to determine that the myosin prepared was indeed cytoplasmic and not derived from possible contamination with intestinal smooth muscle (Figure 2). The antibody recognized purified smooth muscle myosin from turkey gizzard and intestine but failed to recognize the brush border myosin.

The digestion patterns of brush border myosin with both chymotrypsin and papain were very similar to that of human platelet myosin, and the purified fragments had virtually identical SDS–polyacrylamide gel patterns (data not shown).

Characterization of the Actin-Activated MgATPase Activity of Cytoplasmic Myosin Fragments. We first examined the effect of actin on the MgATPase activity of both phospho-

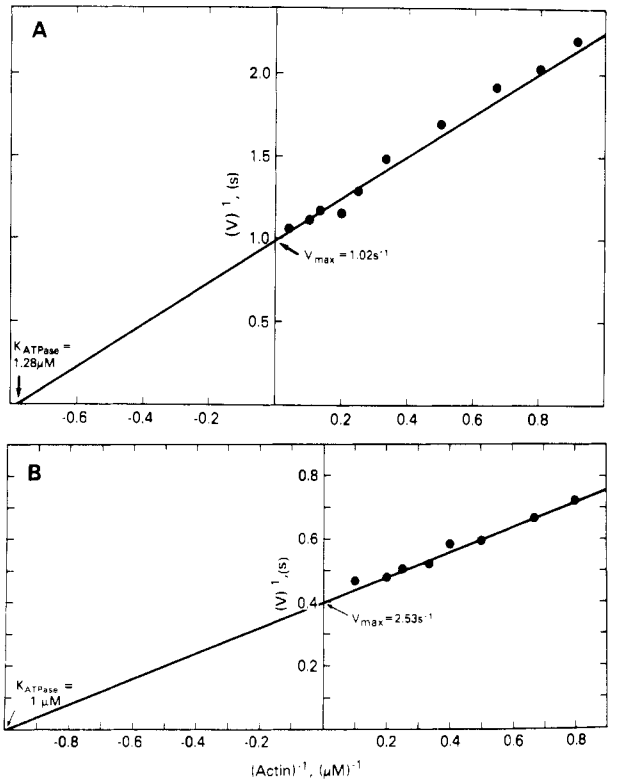

FIGURE 3: Effect of actin on the MgATPase activity of phosphorylated platelet and brush border HMM. Conditions were 10 mM imidazole (pH 7.0), 2 mM $MgCl_2$, 1 mM ATP, 0.1 mM EGTA, and 0.5 mM dithiothreitol, 25 °C. The MgATPase activity of phosphorylated HMM in the absence of actin was subtracted from each data point. The data were computer fitted to the Michaelis–Menten equation by nonlinear regression using the Marquardt compromise. (Panel A) MgATPase activity of phosphorylated platelet HMM; (panel B) MgATPase activity of phosphorylated brush border myosin.

rylated human platelet HMM and turkey brush border HMM (Figure 3). In both cases, the V_{max} was relatively similar to that of turkey gizzard HMM, but the K_{ATPase} values of the cytoplasmic myosins were very low (about 1–2 μM compared to a value of about 40 μM for gizzard HMM).

The analysis of the data of four separate preparations of platelet HMM gave an average value of V_{max} of 0.88 ± 0.35 s^{-1} and a K_{ATPase} of 2.1 ± 0.81 μM. While there was some variability in the values of V_{max} and K_{ATPase} from preparation to preparation the data shown are representative for HMM preparations from both sources.

We next examined the effect of actin on the MgATPase activity of platelet S-1 and found a K_{ATPase} of 16.7 μM and a V_{max} of 0.67 s^{-1} at 25 °C. While the V_{max} value is very similar to that of turkey gizzard S-1, the K_{ATPase} is lower.

Binding of Phosphorylated Platelet and Brush Border HMM to Actin in the Presence of ATP. The low value of K_{ATPase} suggested that the binding of the phosphorylated HMM to actin was very strong. This was examined in a binding assay performed under similar conditions as those used to measure the MgATPase activity (Figure 4). The results indicate that the binding of phosphorylated cytoplasmic myosin to actin is indeed strong with a K_D of 1.32 μM. It should be noted that the binding data extrapolated to a maximal fraction bound of only 0.83 instead of 1.0, suggesting that there is possibly a component present with a weaker binding constant. However, we did not feel the data were precise enough to merit a multicomponent analysis.

Effect of Ionic Strength on the Actin-Activated MgATPase Activity of Phosphorylated Platelet HMM. It was of interest to determine the effect of increasing ionic strength on the

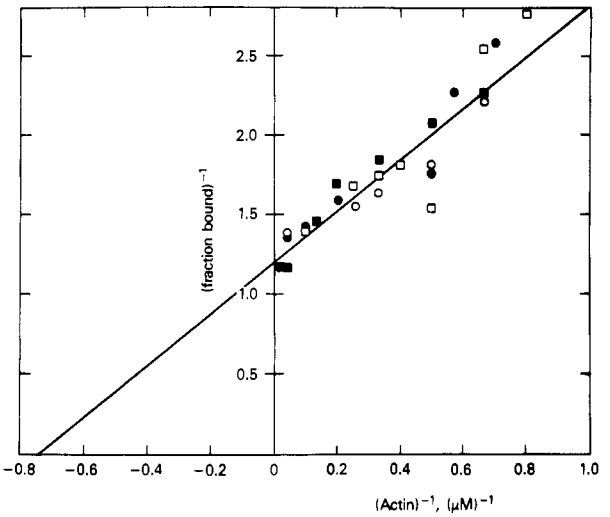

FIGURE 4: Binding of phosphorylated platelet and brush border HMM to actin in the presence of ATP. Conditions were the same as in Figure 3 except that 1 mg/mL bovine serum albumin was present in all cases. The binding assay was performed as described under Experimental Procedures. (●, □, ○) Different experiments with different preparations of phosphorylated platelet HMM; (■) experiment with phosphorylated brush border HMM. The data were computer fitted by a program that determines both the K_D and the extrapolated binding at infinite actin. Only the platelet data were used in the fitting.

actin-activated MgATPase activity of phosphorylated platelet HMM. Increasing the ionic strength from 12 to 112 mM has only a small effect on V_{max} (0.51 to 0.65 s^{-1}) with a larger increase in the value of K_{ATPase}. The values of K_{ATPase} at 12, 62, and 112 mM ionic strength were found to be 1.3, 2.8, and 7.7 μM, respectively. This represents only a 6-fold increase over a very large range of ionic strengths.

Discussion

Most of the extensive knowledge of the kinetic mechanisms of the interaction of vertebrate skeletal and smooth muscle myosin with actin has been obtained by using the soluble and enzymatically active fragments heavy meromyosin (HMM) and subfragment 1 (S-1) (Adelstein & Eisenberg, 1980). To date, almost nothing is known of the mechanism of interaction of cytoplasmic myosins with actin. In order to facilitate these types of studies, we have described the preparation of HMM and S-1 from myosin purified from both human platelets and avian intestinal brush border. In addition, we have begun to characterize the steady-state kinetic properties of these fragments.

There are qualitative similarities between the digestion patterns of smooth muscle myosin and these cytoplasmic myosins. In both cases, papain digestion results in the production of S-1 that is missing its 20-kDa light chain, and chymotryptic digestion produces predominantly HMM (Sellers et al., 1981; Seidel, 1980; Greene et al., 1983). All types of vertebrate myosins have a proteolytically sensitive region (site III) located at about 70–75 kDa from the amino terminus of the molecule (Sellers & Harvey, 1984; Schneider et al., 1985; Okamoto & Sekine, 1978; Marianne-Pepin et al., 1983; Applegate et al., 1984; Yamamoto & Sekine, 1980; Mornet & Morales, 1984). Digestion at site III does not result in dissociation of the cleaved polypeptides under native conditions. Both of the cytoplasmic myosins examined in this study are cleaved very rapidly at this position by papain and chymotrypsin. This makes it difficult to prepare the fragments with intact heavy chains. In this regard, we have found that actin protects the myosin from cleavage at site III (data not shown). This may prove useful in purifying intact fragments in the

future. Cleavage at site III does not appear to have a major effect on the actin-activated MgATPase of turkey gizzard HMM, although the effects of this digestion on the K_{ATPase} and V_{max} were not determined (Margossian et al., 1984).

The S-1 and HMM prepared from these two cytoplasmic myosins both had V_{max} values similar to those reported previously for the fragments prepared from turkey gizzard myosin (Ikebe & Hartshorne, 1985; Greene et al., 1983; Sellers et al., 1982). In all cases, a simple hyperbolic dependence of the MgATPase activity on actin concentration was observed. The surprising feature of these experiments was the extremely low values for the K_{ATPase} that were determined. The average value of about 2 μM for the K_{ATPase} for HMM from brush border and platelet is about 20 times less than that determined for HMM from turkey gizzard smooth muscle under the same conditions (Sellers et al., 1982). Direct measurements of the binding of platelet and brush border HMM to actin gave a K_D of 1.3 μM which is in good agreement with the K_{ATPase} value. The reason for the small variation in the values of K_{ATPase} from preparation to preparation may be related to small, but variable, amounts of S-1 (S-1 has a higher K_{ATPase} value than does HMM) present or to variable losses of the 20 000-Da light chain. It is not known how the loss of the 20 000-Da light chain from one head of HMM would affect the actin-activated MgATPase activity of the other head. The actin dependence of the MgATPase activity (K_{ATPase}) and the binding of cytoplasmic HMM to actin ($K_{binding}$) show similar values. This behavior was also observed with smooth muscle HMM (Sellers et al., 1985) but is in contrast to the skeletal system where the actin-activated MgATPase saturates at lower actin concentration than does the binding of HMM to actin (Stein et al., 1984).

The apparently strong binding of cytoplasmic HMM to actin may be in part explained by the relative tight binding of cytoplasmic S-1 to actin (K_{ATPase} = 16 μM). Under most conditions in the presence of ATP, HMM (from skeletal muscle) has been shown to bind via only one head to actin at a time since the binding constant of the second head is reduced. Greene empirically determined that above a single-headed binding constant of about 10^4 M^{-1} (K_D = 100 μM), the second head contributes very little to the binding constant for HMM (Greene, 1981). As the single-headed binding constant becomes larger (smaller K_D), however, the binding of the second head becomes more important. Using her formulation, one would predict a K_D of 2.8 μM for the cytoplasmic HMM based on the value of 16.7 μM for the K_{ATPase} of S-1 determined in this study. This value is very close to the experimentally determined average K_{ATPase} of 2.1 μM for platelet HMM.

While the implications of this strong binding are not known, it is tempting to speculate that it may be related to the lower concentrations of actin found within the cytoplasm of nonmuscle cells compared to that found in muscle cells. Thus, the increased binding constant may be compensatory to assure that cytoplasmic myosins can interact with actin even though in the cell there is no well-defined sarcomeric structures in which high local actin concentrations may be achieved.

The K_{ATPase} of platelet HMM is not greatly increased by increasing the ionic strength from 12 to 112 mM. In this regard, the behavior of the cytoplasmic HMM seems to be similar to S-1 and HMM from smooth muscle and in contrast to the fragments from skeletal muscle myosin whose binding to actin in the presence of ATP is greatly weakened by relatively small increases in the ionic strength (Table I) (Chalovich et al., 1984). Greene et al. (1983) showed that the K_{ATPase} for smooth muscle S-1 was increased about 19-fold over this range of ionic strengths and the K_{ATPase} measured at 112 nM ionic strength was 312 μM. The value of K_{ATPase} for platelet HMM under these conditions is 7.7 μM.

Table I: K_{ATPase} of Myosin Fragments from Various Muscles and the Effect of Ionic Strength

sample	ionic strength (mM)	K_{ATPase} (μM)	ref
bovine cardiac muscle S-1[a]	16	37	Tobacman & Adelstein (1986)
rabbit skeletal muscle S-1[b]	12	23	Chalovich et al. (1984)
rabbit skeletal muscle S-1[b]	50	137	Chalovich et al. (1984)
turkey smooth muscle S-1	12	32	Greene et al. (1983)
turkey smooth muscle S-1	72	71	Greene et al. (1983)
turkey smooth muscle S-1	112	590	Greene et al. (1983)
human cytoplasmic S-1	12	17	this report
rabbit skeletal muscle HMM[a]	21	25	Wagner & Stone (1983)
turkey smooth muscle HMM[c]	12	43	Sellers et al. (1982)
turkey smooth muscle HMM[c]	173	330	Greene & Sellers (1987)
human cytoplasmic HMM[c]	12	1.3	this report
human cytoplasmic HMM[c]	62	2.5	this report
human cytoplasmic HMM[c]	112	7.7	this report

[a] Measured in the presence of troponin–tropomyosin and Ca^{2+}. [b] Measurement made with the S-1(A1) isoenzyme only. [c] Measurement made with fully phosphorylated HMM.

In the absence of ATP, HMM or S-1 binds to actin with a very high affinity to give a specific arrowhead or chevron appearance when negatively stained and viewed by electron microscopy. Three-dimensional reconstruction of these structures has yielded information about the structure of myosin heads and actin, and about the angle of attachment of myosin to actin. Since ATP binding greatly reduces the affinity of myosin for actin, it has been impossible to study the attachment of myosin to actin in the presence of ATP by this technique using fragments from smooth or skeletal muscle. However, since the binding constant of platelet HMM to actin in the presence of ATP is 20 times stronger than that of skeletal muscle, it should be possible to directly visualize the binding of this myosin fragment to actin in both the presence and absence of ATP.

In conclusion, we have prepared the enzymatically active soluble fragments HMM and S-1 from both human platelet and avian epithelial brush border myosin. We have found that the respective fragments from these two cytoplasmic myosins show very similar properties, judging from their proteolytic digestion patterns and their steady-state kinetics. In each of these cases, there are important quantitative differences between the behavior of the two cytoplasmic myosins (and their fragments) and that of turkey gizzard smooth muscle myosin. The cytoplasmic fragments can be produced in milligram quantities and are stable for at least a week if stored on ice. They hopefully will facilitate future studies on the regulation

and kinetic mechanisms of cytoplasmic myosins.

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