Cooperativity and Regulation of Scallop Myosin and Myosin Fragments†

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ABSTRACT: Scallop heavy meromyosin (HMM) preparation obtained by a new improved method showed a Mg-ATPase activity that was activated 15-fold by calcium. The ATPase activity depended on ionic strength and reached maximum at 0.1 M without altering calcium sensitivity. The highly regulated HMM and myosin preparations showed cooperative properties not seen with unregulated subfragment 1 (S1). ATPase activity of myosin and HMM increased steeply with calcium concentration, yielding Hill coefficients about 3 and 4, respectively. Calcium binding by HMM and myosin became cooperative in the presence of ADP, AMP-PNP, or ADP·Vi yielding Hill coefficients of 1.8 and 2.8, respectively. Binding of calcium by HMM in the presence of ATP was also cooperative at physiological ionic strength, whereas at low ionic strength the data fit best to a simple binding curve. In contrast, calcium binding by unregulated S1 followed a normal binding curve and was not affected by the presence of nucleotide analogues. Calcium decreased the affinity of ADP and ADP-PNP to myosin and HMM, but had no effect on the nucleotide binding to S1. The results indicate that communication between the nucleotide and calcium binding sites requires the presence of two heads and exists only in the "off" state. We propose that in the presence of calcium, interaction between the two heads is disrupted and they act independently.

Muscles are activated when the sarcoplasmic calcium concentration rises from the resting level of about 10 nM to about 1 μM. In the absence of calcium, muscle functions are inhibited. The mechanism of activation varies with muscle type. The "on" and "off" switch of skeletal muscle is the troponin−tropomyosin system associated with the thin filaments (*1*). Binding of calcium to troponin C leads to a series of events that changes the interaction of myosin with actin and results in contraction. Unlike skeletal muscle myosins, smooth and molluscan myosins are regulated molecules. Smooth muscle myosin is triggered by phosphorylation of its regulatory light chain (RLC)[1] by a calcium−calmodulin-dependent myosin light chain kinase, and molluscan myosins are regulated by direct binding of calcium to domain I of their essential light chain (ELC) (reviewed in refs *2* and *3*).

One of the features all muscles share is the cooperative activation following the release of calcium into the cytoplasm. Troponin C binds calcium cooperatively, yielding a Hill coefficient of 2.4 when bound to actin−tropomyosin in the presence of myosin (*4*). Tension is also developed cooperatively with a Hill coeffficient of approximately 5 (*5*), probably mediated by troponin I and tropomyosin on several actin sites (*6*, *7*). Studies with smooth muscle HMM have suggested that the heads are phosphorylated randomly, and that phosphorylation of both heads is required for the activation of the Mg-ATPase activity of either head by actin (*8*, *9*). The dependence of tension generation by skinned scallop muscle fiber bundles and the actin-activated ATPase on calcium were very similar and highly cooperative processes, displaying Hill coefficients of about 4.5 (*10*, *11*). The ATPase activity of scallop HMM, which is also regulated by calcium, was activated cooperatively, yielding a Hill coefficient of about 2. However, the saturation of scallop myosin and HMM with calcium showed a hyperbolic dependence on calcium, even in the presence of ATP (*10*). Recently, we showed that single-headed myosin is a partly regulated molecule, the activation of which by calcium follows a simple binding curve (*12*).

Molluscan myosin is particularly suitable for studying the relationship between the calcium and nucleotide binding sites, since activation results from binding of a simple ligand. We have re-examined the question of calcium binding and calcium activation by the regulated scallop myosin and scallop HMM and by the unregulated subfragment 1 (S1), using improved preparations of HMM and S1. We show that in the presence of nucleotides, the regulated scallop myosin and scallop HMM bind calcium cooperatively. The heads of the regulated molecules bind nucleotides independently in the presence of calcium, but cooperatively in its absence. Furthermore, calcium and nucleotides bound antagonistically to the regulated myosin and HMM, but bound independently to the unregulated S1. We suggest that the interactions between the two heads which are responsible for the regulation of scallop myosin also give rise to the cooperative binding of its ligands.

EXPERIMENTAL PROCEDURES

Protein Preparations. Scallop myosin was prepared from *Argopecten irradians* according to Stafford et al. (*13*). S1 was prepared by digesting myosin with freshly prepared affinity-purified papain (*14*). Myosin was suspended at 5−10 mg/mL in 20 mM MOPS, 60 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 3 mM NaN_3, 0.5 mM DTT, pH 7.0 (buffer A), and digested with papain at 20 °C for 10 min, using a

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[1] Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP-PNP, adenylyl imidodiphosphate; Vi, vanadate ion; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; NaDod SO_4, sodium dodecyl sulfate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NADH, α-dihydronicotinamide adenine dinucleotide; S1, subfragment 1; HMM, heavy meromyosin; LMM, light meromyosin; RLC, regulatory light chain; ELC, essential light chain.

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w/w ratio of myosin to papain of 2000. The digestion was stopped by the addition of leupeptin at 20 μg/mL. The digestion mixture was cooled on ice and clarified by centrifugation at 40 000*g* for 30 min. The supernatant was applied to a 10 × 1.5 cm column of reactive red 120 (Sigma), and the column was washed with 5 bed volumes of buffer A supplemented with 5 μg/mL leupeptin. Bound proteins were eluted by stepwise increase of KCl concentration of buffer A to 2 M. Samples of 1−2 mL were collected manually. S1 eluted with the void volume and was concentrated by precipitation with saturated ammonium sulfate to 60%, followed by dialysis against buffer A containing 5 μg/mL leupeptin. To prepare HMM, 4 g of myosin (10 mg/mL) was digested with trypsin (5 units/mg of myosin) in 10 mM P_i, 0.6 M NaCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.1 mM EDTA, 3 mM NaN_3, 0.5 mM DTT, pH 6.8, for 2 min at 20 °C. The digestion was stopped by the addition of soybean trypsin inhibitor at 10 mg/mg of trypsin. The digestion mixture was dialyzed against 10 mM P_i, 40 mM NaCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 3 mM NaN_3, 0.5 mM DTT, pH 7.0 (buffer B). The sample was clarified by centrifugation at 40 000*g* for 30 min, and then it was applied at a rate of 3 mL/min to a 16 × 1.5 column of Toyopearl DEAE 650 S equilibrated in buffer B. Gradient was established in a Pharmacia FPLC system with a flow rate of 2 mL/min and 2 mL fractions were collected. The gradient must be very slow between 50 and 200 mM NaCl (240 mL), where most of the material is eluted. Fractions having ATPase activity that was activated at least 10-fold on addition of calcium were pooled and dialyzed against 50% glycerol, 0.1 M KCl, 20 mM MOPS, 2 mM $MgCl_2$, 0.1 mM EDTA, 3 mM NaN_3, 1 mM DTT, pH 7.0. The HMM preparation was kept at −20 °C and was used within 1 week.

Measurements of ATPase Activity. The ATPase activities of S1, HMM, and myosin were measured with the coupled assay method at 22 °C. The solution contained 1 mM ATP, 2 mM $MgCl_2$, 1 mM phosphoenolpyruvic acid, 0.15 mM NADH, 10 units of pyruvate kinase, 20 units of lactic dehydrogenase, 0.2 mM EGTA, 100 mM KCl, 20 mM MOPS−KOH, pH 7.0, and various concentrations of $CaCl_2$ to obtain the desired concentration of free calcium. To measure actin-activated ATPase activities, the KCl was omitted from the assay medium, and the concentration of MOPS−KOH was decreased to 5 mM. The decrease of the pH during the assay never exceeded 0.05 pH unit. The oxidation of NADH was monitored at 340 nm with a Perkin-Elmer 552A spectrophotometer fitted with a thermostated cell holder. At ionic strength of 0.1 M, the turnover rates of S1, HMM, and myosin per head were in the range of 0.35−0.45 mol^{-1} s^{-1}.

Binding Assays. Binding of ^{45}Ca, [^{3}H]AMP-PNP, and [^{3}H]-ADP to the soluble S1 and HMM was measured with the method of Hummel and Dreyer (*15*). Calcium binding to myosin was measured according to Chantler et al. (*10*), except the included volume was determined by weighing the centrifuged pellet. To measure the binding of AMP-PNP and ADP to myosin, the tritium-labeled nucleotide was added to a suspension of myosin, and after the sample was incubated for 15 min the filaments were pelleted by centrifugation. The concentration of bound and free nucleotide was determined by comparing the amount of radioactivity in an aliquot of the sample prior to centrifugation, in the supernatant, and in the pellet containing the myosin filaments. The measurements were carried out at room temperature (22 ± 1 °C). To measure binding of AMP-PNP or ADP, the solution contained 20 mM MOPS, 40 mM KCl, 10 mM $MgCl_2$, 3 mM NaN_3, 0.5 mM DTT, and 1 mM EGTA or 0.1 mM $CaCl_2$, and various concentrations of the nucleotide at pH 7.0. When binding of ADP was measured, the solution contained the 5 μM diadenosine pentaphosphate. Calcium binding was measured in the same buffer solution except the concentration of ^{45}Ca was kept constant at 0.1 mM and the concentration of EGTA was varied to obtain the desired concentration of free calcium. Free calcium concentrations were calculated using a program that uses an iterative procedure (*16*). Binding was measured on 30−40 μL of ∼2 mg/mL protein samples.

Data Analysis. The line through the data points of the plots was drawn empirically. However, the Hill coefficient was calculated from the slope of the Hill plot which was linear between 15% and 85% saturation. Linear least-squares analysis of this line gave the Hill coefficient. The standard error of the Hill coefficient was typically ±5% of the average. All binding curves were normalized. With cooperative binding the binding curve always reached a plateau (100% saturation). In some cases of noncooperative binding saturation was not reached and the saturation point was found by fitting the data to a hyperbola. The experimentally determined saturation was set to 1. The saturation (moles of bound ligand per moles of binding sites) was always greater than 70% of the theoretical maximum.

Purification of Radioactively Labeled Nucleotides. Radioactively labeled nucleotides contained radioactive material that did not bind to myosin. This was especially significant for [^{3}H]AMP-PNP. Therefore, the nucleotides were purified by binding to excess myosin; the purified radioactive nucleotide was then displaced with the aid of unlabeled nucleotides. The purified preparation bound completely to myosin, HMM, and S1. All steps were carried out at 4 °C. [^{3}H]AMP-PNP (ICN) and [^{3}H]ADP (NEN) were added to scallop myosin suspended at 2 mg/mL in 10 mM MOPS, 20 mM KCl, 1 mM $MgCl_2$, 0.1 mM EGTA, to yield a molar ratio of nucleotide to myosin of 1:10. After the sample was incubated for 5 min, the myosin filaments were collected by centrifugation at 5000*g* for 5 min and were washed once in the same buffer. The final pellet was suspended in 10 mM MOPS, 20 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.05 mM unlabeled nucleotide. Following a 20 min incubation, myosin was pelleted at 5000*g* for 5 min; the supernatant was saved, and the pellet was washed by centrifugation in the same buffer. The supernatants contained the purified tritiated nucleotide and were stored at −20 °C. The concentration of the nucleotides was determined spectrophotometrically using an extinction coefficient of 15 400 M^{-1} cm^{-1} at 259 nm.

Procedure for the Formation of the Myosin·ADP·Vi Complex. The myosin·ADP·Vi complex was formed by incubating myosin (2 mg/mL) in 40 mM KCl, 20 mM MOPS, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.5 mM ADP, 0.2 mM vanadate, 0.5 mm DTT, 3 mM NaN_3, 5 μM diadenosine pentaphosphate, pH 7.0, for 30 min at room temperature. Calcium was included because the formation of the ternary complex of myosin·ADP·Vi proceeds faster in its presence. Monomeric vanadate was prepared as described by Goodno (*17*).

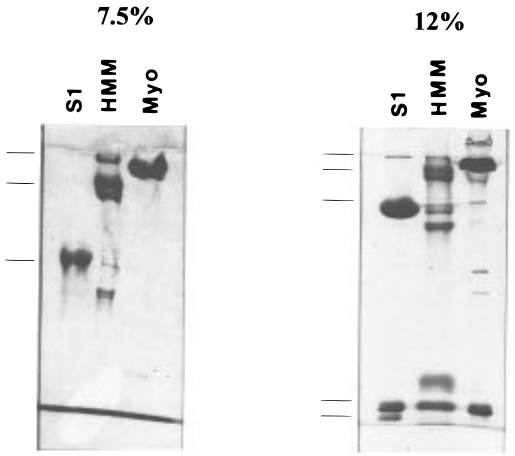

FIGURE 1: Electrophoretic profile of S1, HMM, and myosin. $NaDodSO_4$-polyacrylamide gels (7.5% and 12%) were run in the Laemmli system. All proteins contained stoichiometric amounts of heavy chains and light chains. The S1 contained a single heavy chain, an intact ELC, and a partly clipped RLC. The HMM contained two major heavy chain fragments and intact light chains. Different HMM preparations are shown in the two panels. The left panel shows a "good" HMM preparation and the right panel shows a somewhat overdigested preparation. Note the extra bands between the light chains and the heavy chains in the HMM preparation shown in the right panel. The band running just above the light chains is the trypsin inhibitor. Dashes indicate the position of myosin, HMM, S1, light chains, and clipped RLC.

RESULTS

Characterization of Scallop S1 and Scallop HMM. Scallop S1 and scallop HMM were prepared by proteolytic digestion of scallop myosin with papain and trypsin, respectively. Both preparations contained equimolar amounts of light and heavy chains (Figure 1). In the past, the papain digestion was stopped by the addition of iodoacetic acid. However, this treatment modifies the scallop S1 and gives preparations having ~3-fold higher specific activity. Leupeptin, the potent reversible inhibitor of papain, was used to stop the digestion in order to avoid the modification of scallop S1. To prepare scallop HMM having high calcium sensitivity, the digestion time had to be kept short, consequently lowering the yield of the preparation. About 2 mg of HMM was obtained from 1 g of myosin. The preparation could be improved further by anion exchange chromatography, since the unregulated scallop HMM (generated by nicking of the heavy chains by trypsin) has different chromatographic behavior than the regulated molecules. Separation of the regulated from the unregulated molecules was incomplete, and the calcium sensitivity of the various fractions had to be measured before the fractions could be pooled. Storage of the scallop HMM in 50% glycerol, 0.1 M KCl, 20 mM MOPS, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, pH 7, at −20 °C, protected its ATPase activity and calcium sensitivity for at least 1 month. However, the apparent K_M for ATP of the calcium-activated ATPase activity increased with time. This deterioration of the preparation necessitated its use within 1 week. Quality of HMM preparation was judged by the extent of the ATPase activation by calcium and by the limited extent of degradation of the heavy chain detected on $NaDodSO_4$ gels.

Calcium Activation of the Mg-ATPase Activity of HMM and Myosin. The ATPase activity of HMM and myosin increased steeply with the concentration of free calcium, yielding calcium-activation curves with Hill coefficients ~3 and ~4, respectively (Figure 2, Table 1). The steepness of the calcium-activation curve of the ATPase activity of HMM varied with the quality of the HMM as judged by the calcium sensitivity of the preparation (% calcium sensitivity = {[activity in calcium] − [activity in EGTA]/activity in calcium} × 100). The Hill coefficients varied from ~2 for 75−80% calcium-sensitive preparations to ~3.2 for preparations having over 94% calcium-sensitive ATPase activity. Actin activated the ATPase activity of HMM about 70-fold without affecting the calcium dependence of the activity (data not shown).

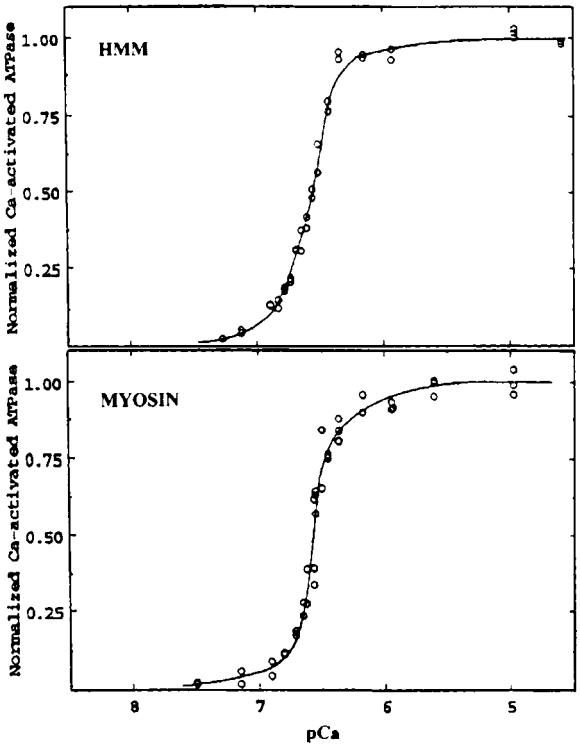

FIGURE 2: Calcium dependence of the calcium-activated Mg-ATPase activity of HMM and myosin. The ATPase activity of HMM (upper panel) and myosin (lower panel) was measured with the coupled assay method in 100 mM KCl, 20 mM MOPS, 2 mM $MgCl_2$, 1 mM ATP, 0.4 mM EGTA, and various concentrations of $CaCl_2$, pH 7.0, at 22 °C. Both HMM and myosin were activated cooperatively with Hill coefficients 2.9 and 3.7, respectively. Half-maximal activation was at 0.25 μM of free calcium for HMM, and 0.27 μM of free calcium for myosin.

Effect of Ionic Strength on the Mg-ATPase Activity and Calcium Sensitivity of the HMM ATPase. The effect of ionic strength on the calcium-activated ATPase activity and calcium sensitivity was examined in the range from 10 to 400 mM. The calcium-activated ATPase activity of scallop HMM increased with the ionic strength to reach a maximum at near physiological ionic strength conditions (Figure 3). The calcium sensitivity of the HMM preparations remained constant at ionic strengths up to ~170 mM and began to decrease at higher ionic strength (Figure 3). The ionic strength up to 160 mM did not affect the steepness or the calcium dependence of the calcium activation curve (data not shown).

Calcium Binding by S1, HMM, and Myosin. The binding of calcium by the unregulated S1 and the regulated HMM and myosin was measured in the absence of nucleotides, as well as in the presence of saturating concentrations of ADP, AMP-PNP, ADP·Vi, and ATP. In the absence of nucleotides, binding of calcium by S1, HMM, and myosin

Table 1: Results of Ligand Binding by S1, HMM, and Myosin[a]

proteins	ligand	[ligand] at half-saturation (μM)	Hill coeff
S1	Ca^{2+}	0.12	1.0
S1 (AMP-PNP)	Ca^{2+}	0.12	1.0
S1 (Ca)	ADP	13.3	1.0
S1, EGTA	ADP	13.3	1.0
S1 (Ca)	AMP-PNP	0.27	1.0
S1, EGTA	AMP-PNP	0.27	1.0
HMM	Ca^{2+}	0.27	1.0
HMM $(ADP)_2$	Ca^{2+}	0.7	1.8
HMM $(AMP\text{-}PNP)_2$	Ca^{2+}	0.8	1.9
HMM $(Ca)_2$	ADP	13.0	1.0
HMM, EGTA	ADP	2.4	1.0
HMM $(Ca)_2$	AMP-PNP	0.28	1.0
HMM, EGTA	AMP-PNP	0.12	1.3
myosin	Ca^{2+}	0.16	1.0
myosin $(ADP)_2$	Ca^{2+}	0.4	2.8
myosin $(AMP\text{-}PNP)_2$	Ca^{2+}	1.0	2.8
myosin $(ADP{\cdot}V_1)_2$	Ca^{2+}	1.0	2.8
myosin $(Ca)_2$	ADP	13.7	1.0
myosin, EGTA	ADP	2.10	1.7
myosin $(Ca)_2$	AMP-PNP	0.52	1.0
myosin, EGTA	AMP-PNP	0.056	1.8
HMM, ATP (40 mM ionic strength)	Ca^{2+}	2.5	1.0
HMM, ATP 170 mM ionic strength)	Ca^{2+}	0.5	1.6

[a] The Hill coeffcients at mid-saturation were determind by the slope of the plot of log [fractional saturation/(1 − fractional saturation)] versus the log [free ligand]. In some titrations for which the end point was not reached, the data could be fit to a simple binding curve, and in these cases experimental points were obtained at ligand concentrations up to $4K_d$ or higher.

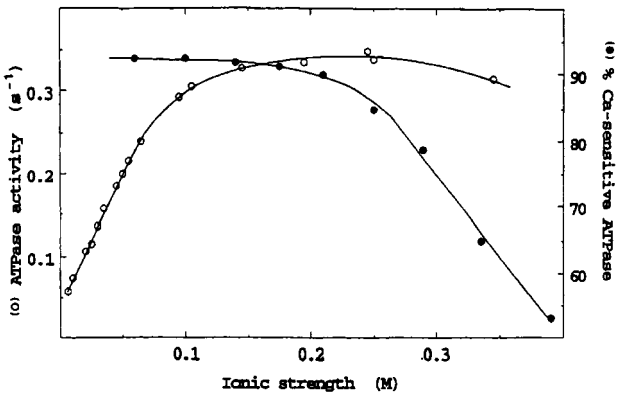

FIGURE 3: Effect of ionic strength on the calcium-activated Mg-ATPase activity and calcium sensitivity of scallop HMM. The ATPase activity of HMM (○) was measured with the coupled assay method in 10 mM MOPS−KOH, 2 mM $MgCl_2$, 1 mM ATP, 0.4 mM EGTA or 0.4 mM EGTA and 0.44 mM $CaCl_2$, pH 7.0, at 22 °C. The ionic strength was varied by changing the concentration of KCl. The calcium sensitivity (●) was calculated from the ATPase activity in the absence and presence of calcium (% calcium sensitivity = $[ATPase^{Ca} - ATPase^{EGTA}]/[ATPase^{Ca}] \times 100$).

followed a simple binding isotherm. All proteins bound calcium with similar affinity (Figure 4, Table 1). In the presence of ADP, AMP-PNP, or ADP·Vi, the regulated HMM and myosin bound calcium cooperatively, whereas the affinity of the unregulated S1 for calcium was unaffected (Figure 4, Table 1). The Hill coefficients for calcium binding by HMM and myosin were typically ∼1.8 and ∼2.8, respectively. The structure of the nucleotide affected calcium binding by shifting the binding curve without affecting its steepness (Figure 4, Table 1). The binding of calcium by HMM in the presence of ATP depended strongly on the ionic strength. At pH 7.0 and 40 mM ionic strength, the best fit to the data was a simple binding isotherm with half-maximal

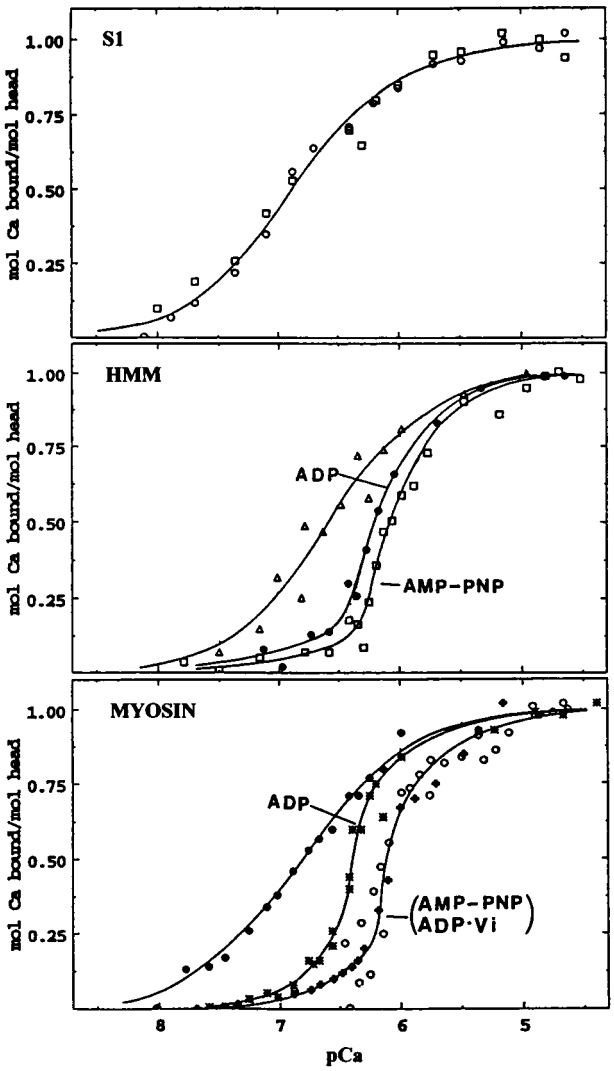

FIGURE 4: Calcium binding by scallop S1, scallop HMM, and scallop myosin. The binding of calcium by S1 (upper panel) was not affected by the presence of saturating concentrations of AMP-PNP (0.2 mM). S1 bound calcium with a K_d of 0.12 μM both in the presence (□) and absence (○) of nucleotide. The calcium binding properties of HMM (middle panel) depended on the nature of the nucleotide. In the absence of nucleotides (△) HMM bound calcium hyperbolically with a K_d of 0.27 μM. In the presence of 1 mM ADP (●) or 0.2 mM AMP-PNP (□) the binding of calcium was cooperative with a Hill coefficient of ∼1.8. Half-maximal binding of calcium occurred at 0.7 μM in the presence of ADP and 0.8 μM in the presence of AMP-PNP. Binding of calcium by myosin (lower panel) was hyperbolic ($K_d = 0.16\ \mu$M) in the absence of nucleotides (●). In the presence of 1 mM ADP (*) or 0.2 mM AMP-PNP (○) or with the ternary complex of myosin with ADP and vanadate (+), myosin bound calcium cooperatively with a Hill coefficient of ∼2.8. In the presence of ADP half-maximal saturation was at 0.4 μM free calcium, whereas in the presence of AMP-PNP or ADP + Vi half-maximal saturation occurred at 1 μM free calcium.

binding at 2.5 μM free calcium (Figure 5). However, at 170 mM ionic strength the binding of calcium was cooperative, and half-maximal binding occurred at 0.5, μm free calcium (Figure 5, Table 1).

Binding of ADP and AMP-PNP by S1, HMM, and Myosin. The binding of [^{3}H]ADP and [^{3}H]AMP-PNP to S1, HMM, and myosin was studied in the presence and absence of calcium.

In the presence of calcium, all proteins bound ADP and AMP-PNP with similar affinities (Figure 6, Table 1). The

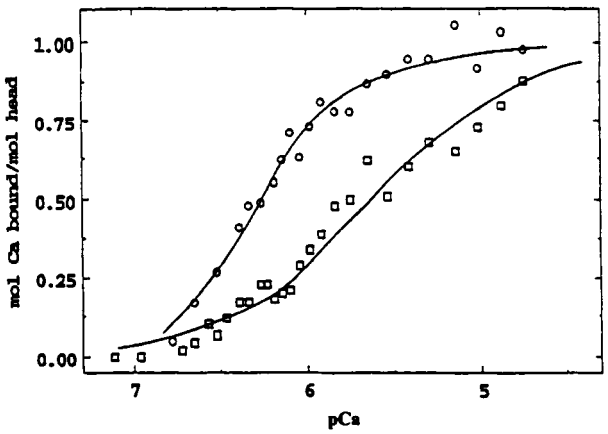

FIGURE 5: Effect of the ionic strength on the calcium binding properties of HMM in the presence of ATP. At 40 mM ionic strength (□) the data were fitted to a simple binding process with an apparent K_d of 2.5 μM. At 170 mM ionic strength (○) binding of calcium by HMM was cooperative with a Hill coefficient of 1.6. Half-maximal binding was at 0.5 μM free calcium.

data were fit satisfactorily to a simple binding isotherm. Removal of calcium from the calcium binding site of S1 did not change its affinity for ADP and AMP-PNP; in contrast, the binding of these nucleotides by myosin and HMM became stronger by calcium removal. In addition, the binding of ADP and AMP-PNP by myosin and binding of AMP-PNP by HMM was cooperative (Figure 6, Table 1), suggesting that the communication between the calcium binding site and the ATP binding site results in the cooperative binding of these nucleotides.

DISCUSSION

The main finding of this paper is that regulation is associated with cooperative events that require interaction between the two myosin heads. Previously, it was shown that the actin-activated ATPase (*10*), tension generation (*11*), and motility (*18*) are activated in a cooperative manner by calcium. We now find that ATPase activity of myosin and HMM is activated in a cooperative fashion, even in the absence of actin, and that calcium binding in the presence of nucleotides becomes cooperative. In addition, the affinity of the nucleotides to regulated molecules is reduced in the presence of calcium. Although the nucleotide and calcium binding sites are present in S1, communication between those two sites is established only by the presence of two heads in regulated molecules, which presumably is necessary to maintain the "off" state.

The experiments necessitated the development of a new improved method for the preparation of scallop HMM in biochemical quantities and allowed for a detailed comparative study of the properties of scallop HMM to its parent scallop myosin molecule. The new HMM preparations were activated by calcium ~15-fold. They contained equimolar amounts of light chains and heavy chains (Figure 1). The light chains were intact and the heavy chains migrated as two closely separated bands. The ATPase activity of the regulated HMM increased with the ionic strength, whereas the ATPase activity of the unregulated scallop S1 was unaffected by changes of ionic strength. In the absence of actin at 0.1 M ionic strength, S1, HMM, and myosin had similar ATPase activities per head at 22 °C (~0.4 mol^{-1} s^{-1}). At 10 mM ionic strength, actin activated the ATPase activity of S1 and HMM ~70-fold.

In the absence of nucleotides, S1, HMM, and myosin bound calcium with similar affinities (Figure 4, Table 1), indicating an absence of interactions between the subunits of these proteins as calcium binds. In the presence of ADP, AMP-PNP, or ADP·Vi, HMM and myosin bound calcium cooperatively with Hill coefficients at mid-saturation of 1.8 and 2.8, respectively. The Hill coefficient of 1.8 for calcium binding by HMM in the presence of saturating concentrations of ADP or AMP-PNP can be attributed to interactions between the heads of scallop HMM, and the calcium binding properties of the protein can be explained by the sequential and concerted models for cooperative behavior (*19*, *20*). However, the high cooperativity of calcium binding by scallop myosin (showing Hill coefficients as high as 2.8) cannot be explained by these models without invoking interactions between myosin molecules within the filaments, because the maximum Hill coefficient is always lower than the number of interacting subunits. An alternative interpretation of the unusually high cooperativity observed with scallop myosin can be based on a particular type of cooperativity analyzed by Weber and Anderson (*21*, *22*), provided that certain conditions are met. The ligand-free and ligand-bound protein must exist predominantly in different conformations. The protein conformation induced by ligand binding has a lower dissociation constant for the binding ligand than the conformation of the ligand-free protein. The rates of ligand binding and dissociation to and from the protein must be considerably faster than the rates of interconversions between the two protein conformations. Electron microscopic studies have shown that the regular arrangement of myosin heads in myosin filaments in the presence of ATP is destroyed by calcium (*23*, *24*). One may assume that the calcium-free heads of scallop myosin associate in the presence of nucleotides and dissociate upon the addition of calcium. It is possible that the binding cooperativity of scallop myosin arising from calcium-dependent head-to-head interactions may be enhanced if the rates of calcium binding and dissociation are different from the rates of the reaction involving the association of the myosin heads.

The binding of calcium by myosin is steeper than the binding of calcium by HMM. This difference may not necessarily be due to the filamentous state of myosin, and very likely depends on the quality of the HMM preparations. This is indicated by the finding that steepness of the activation of ATPase by calcium increased with the calcium sensitivity of the HMM preparation. It is possible to obtain small amounts of HMM having calcium sensitivity greater than 94% by carefully collecting fractions during the anion exchange chromatography, and such preparations were used in studies in which the ATPase activity was monitored with the very sensitive coupled assay method. However, to obtain enough material for the binding studies, it was necessary to sacrifice some of the quality of the HMM preparation in order to increase the yield. The calcium sensitivity of the HMM preparations used in the binding studies was ~90%.

The high Hill coefficients for the activation of the ATPase activity of scallop HMM and scallop myosin by calcium may also be explained by the type of cooperativity described by Weber and Anderson (*21*). A point of interest is the difference between the calcium binding and the activation of the ATPase activity of HMM and myosin by calcium in the presence of saturating concentrations of ATP. At 40 mM ionic strength, the binding of calcium by HMM in the

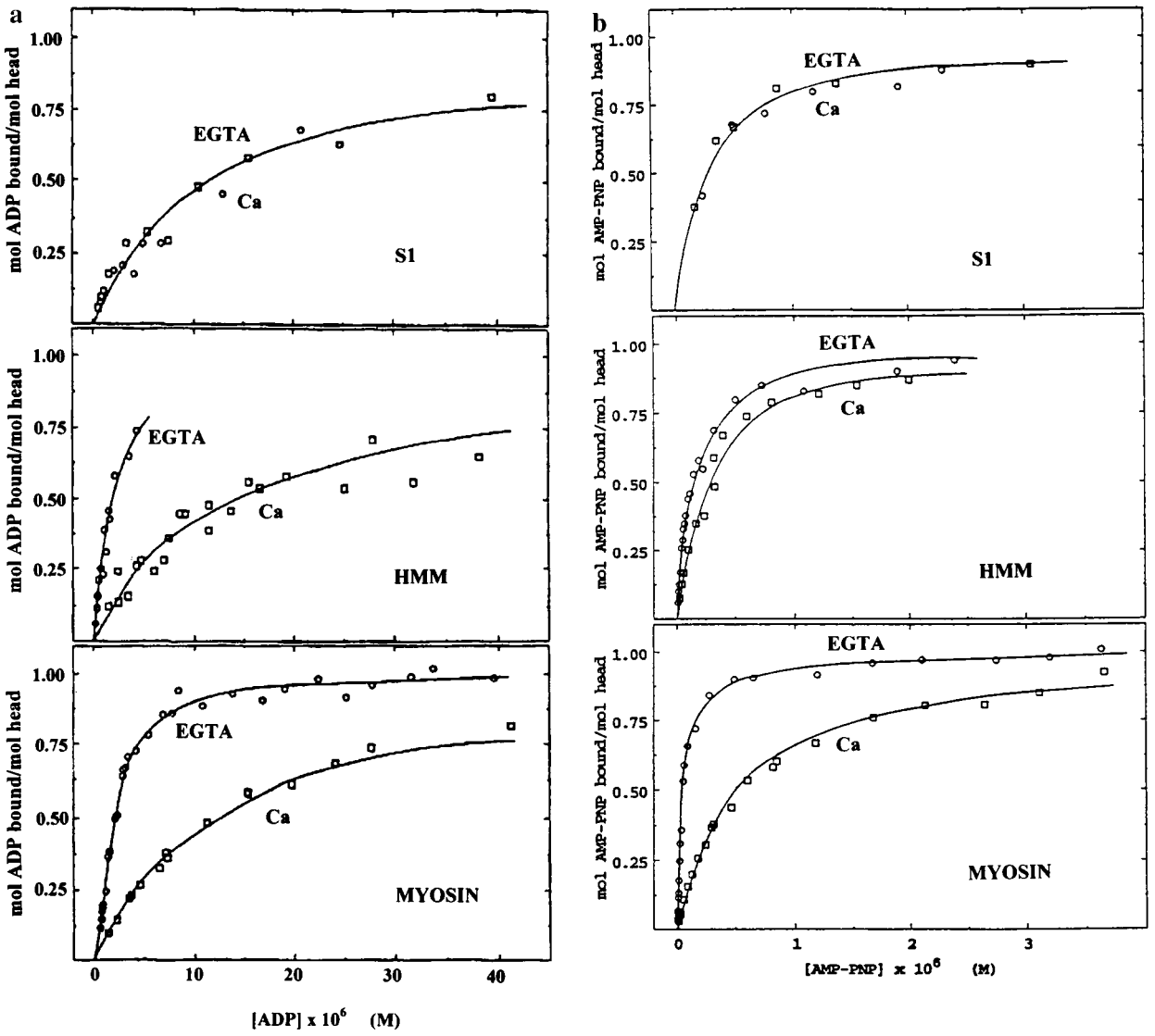

FIGURE 6: (a) Binding of ADP by S1, HMM, and myosin. S1 (upper panel) bound ADP with apparent K_d of 13 μM in both the presence (□) and absence (○) of calcium. HMM (middle panel) bound ADP with an apparent K_d of 13.4 and 2.4 μM in the presence (□) and absence (○) of calcium, respectively. In the presence of calcium (□) myosin (lower panel) bound ADP with an apparent K_d of 13.7 μM. In the absence of calcium (○) binding of ADP was cooperative, yielding a Hill coefficient of 1.7. Half-maximal binding was at 2.1 μM. (b) Binding of AMP-PNP by S1, HMM, and myosin. S1 (upper panel) bound AMP-PNP with an apparent K_d of 0.27 μM in both the presence (□) and absence (○) of calcium. HMM (middle panel) bound AMP-PNP noncooperatively in the presence of calcium (□) with an apparent K_d of 0.28 μM. In the absence of calcium (○) binding was cooperative with a Hill coefficient of 1.3. Half-maximal binding was at 0.12 μM. Myosin (lower panel) bound AMP-PNP noncooperatively in the presence of calcium (□) with an apparent K_d of 0.52 μM and cooperatively in the absence of calcium (○). The Hill coefficient was 1.8 and half-maximal binding occurred at 0.056 μM.

presence of ATP was described best by a single binding isotherm. This observation was in agreement with previous studies of scallop HMM that were carried out under similar conditions (*10*). However, at higher ionic strength (170 mM) the binding of calcium by HMM became cooperative, and the binding curve shifted toward the curve showing calcium activation of the ATPase activity. Figure 4 shows that ADP, AMP-PNP, or ADP·Vi affect the affinity of calcium to HMM or myosin somewhat differently, as shown in three different preparations. This observation suggests that calcium binding by the regulated scallop HMM and scallop myosin depends on the nature of the nucleotide occupying the ATP binding site. Thus, it is possible that the various intermediates occurring during the ATPase cycle bind calcium with different affinities. The overall calcium binding in the presence of ATP arises from the superposition of various binding curves, each one given by a different intermediate, which are displaced from each other along the *x* axis. At low ionic strength, the sum of these curves gives rise to one that is described best by a simple binding curve (Figure 5). Changes in the ionic strength shift the relative populations of the various intermediates of the ATPase cycle. This is evident from the ionic strength dependence of the calcium-activated ATPase activity of HMM shown in Figure 3. Since the ATPase activity of scallop HMM increases with the ionic strength, the latter must influence the proportion of the intermediates accumulating before the rate limiting step(s). The position and the steepness of the calcium-activation curve of the ATPase activity in solution is controlled by very few steps of the reaction cycle. However, when calcium binding is measured, the observed calcium binding represents all the intermediates that bind calcium with varying affinities. The fraction of the intermediates of the reaction cycle varies with the ionic strength, resulting in a shift of the calcium binding curve with the change of the ionic strength (Figure 5).

An interaction between binding sites in a protein is expected to generate an interdependence of the binding properties of the respective ligands for these sites, and the ligands will bind either synergystically or antagonistically. Binding of nucleotides to the unregulated S1 was not expected to be influenced by calcium, in accordance with the experimental observations (Figure 6) indicating a lack of communication between the calcium binding and the ATP binding site in this protein. However, with the regulated scallop HMM and scallop myosin, the binding of nucleotides should be dependent on the occupancy of the calcium binding site by calcium. This is demonstrated in Figure 6. In the presence of calcium the heads of HMM and myosin bound ADP or AMP-PNP independently, since the data could be fit to a simple binding curve. In the presence of EGTA, when the calcium binding sites are empty, both HMM and myosin bound ADP and AMP-PNP with higher affinity than in the presence of calcium. HMM bound ADP noncooperatively and AMP-PNP cooperatively, whereas myosin bound both ADP and AMP-PNP cooperatively (Figure 6, Table 1). The differences in the binding properties of HMM and myosin for ADP and AMP-PNP may be attributed to either some deterioration of the scallop HMM resulting from the experimental manipulations necessary to purify the protein, or an altered strength of the interactions of the heads of HMM due to the proteolytic removal of the LMM part of the myosin (which enables it to assemble into filaments). We have noticed consistently that myosin is activated by calcium more steeply than HMM and that it binds calcium and nucleotides with a higher cooperativity than HMM. A comparison of the various HMM preparations has also suggested that the cooperative behavior exhibited by HMM increases with the quality of the preparation. Thus, the observed differences between HMM and its parent myosin must be due at least in part to the quality of the biochemical preparations of HMM. Another reason for the differences between HMM and myosin can be sought in the relative strengths of the interactions between the heads of the two molecules. In his analysis of cooperativity based on the conservation of free energy, Weber demonstrated that although protein association promoted by ligand binding can change the affinity of the associating subunits for the binding ligand (in the context of scallop myosin the associating proteins are the myosin heads), the binding of the ligand may appear to be noncooperative under certain conditions (*25*).

This work has provided evidence that the binding of nucleotides induces an interaction between the two heads of myosin and HMM that results in a communication between the nucleotide and the calcium binding sites. Addition of calcium probably disrupts the interaction between the myosin heads, and they behave independently of one another; communication between the nucleotide and the calcium binding sites no longer exists. The "on" and "off" states of these proteins represent different structures with different affinities to nucleotides. In the presence of calcium nucleotide binding is significantly weaker and becomes noncooperative (Figure 6, Table 1). The structural basis of this interaction is unclear. It could be that the binding of nucleotides induces an association of the heads which is disrupted by calcium. A highly ordered structure formed by myosin heads in myosin filaments in the "off" state which becomes disordered by calcium has been demonstrated by electron microscopic studies (*23*, *24*). A bending of the HMM heads toward the tail is also a possibility (*26*). Weber has demonstrated that binding of a ligand in a cooperative fashion can promote association of proteins (*25*). (The myosin heads are a special case in that treatment because they are held "together" physically.) A nucleotide-dependent head-to-head interaction is also supported by calcium binding studies (Figure 4, Table 1) because binding of calcium is hyperbolic in the absence of nucleotides and cooperative in their presence. Therefore, regulation depends on interaction between the two heads.

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