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Calcium-Sensitive Binding of Heavy Meromyosin to Regulated Actin Requires Light Chain 2 and the Head–Tail Junction†

Paul D. Wagner* and Deborah B. Stone

ABSTRACT: Sedimentation in a preparative ultracentrifuge was used to determine the affinity of heavy meromyosin, HMM, for regulated actin, F-actin plus troponin–tropomyosin, in the presence of MgATP at pH 7.0, 20 °C, and μ = 18 mM. HMM was prepared from vertebrate striated muscle myosin by a mild chymotryptic digestion. This HMM contained 85–90% intact 19 000-dalton light chains, LC2. In the presence of calcium, 90% of the HMM bound to regulated actin with an association constant of (2–4) × 10^4 M^{-1}. In the absence of calcium, while one-third of the HMM bound with an affinity similar to that observed in the presence of calcium, the rest bound much more weakly. It was not possible to accurately determine the association constant for this weakly binding HMM, but a 20-fold reduction in affinity is consistent with the binding data. The binding of single-headed heavy meromyosin to regulated actin was similarly sensitive to the calcium concentration. Since removal of calcium inhibits the regulated actin-activated ATPase of HMM greater than 20-fold, troponin–tropomyosin must be capable of inhibiting both the binding of HMM to regulated actin and a step which occurs after binding but prior to product release. Removal of LC2 increased the fraction of HMM with calcium-insensitive binding, and adding LC2 back to this depleted HMM restored most of the calcium sensitivity. Chymotryptic cleavage of LC2 to a 17 000-dalton fragment destroyed the calcium-sensitive binding of HMM to regulated actin. Phosphorylation of LC2, however, had no detectable effect on this binding. Thus, the calcium-sensitive binding of HMM to regulated actin requires that both the head–tail junction and the N-terminal part of LC2 be intact. Binding studies with cross-linked regulated actins and kinetic measurements of the rates of change in turbidity demonstrate that this calcium sensitivity is due to calcium binding to troponin and not to LC2.

In vertebrate striated muscles, contraction is regulated by calcium binding to troponin–tropomyosin on the thin filament (Ebashi et al., 1969). In the absence of calcium, troponin–tropomyosin inhibits the cyclic interaction of actin with the myosin cross bridges and, hence, force development. Calcium binding to troponin causes a change in the position of tropomyosin on the thin filament (Huxley, 1972; Haselgrove, 1972; Parry & Squire, 1973), allowing for the myosin cross bridge to interact with actin and force to be developed. It has been thought that relaxed muscle has low stiffness because tropomyosin in the absence of calcium prevents myosin binding to actin either by physically blocking the myosin binding sites on actin (Huxley, 1972; Haselgrove, 1972; Parry & Squire, 1973) or by causing a conformational change in the actin. Differences in X-ray diffraction patterns of relaxed and contracting muscles (Huxley, 1972) and the random orientations of spin-labels (Thomas & Cooke, 1980) and fluorescent dyes (Borejdo & Putnam, 1977) attached to the cross bridges in relaxed glycerinated fibers also indicate that most, if not all, of the myosin cross bridges are dissociated from actin during relaxation. However, recent in vitro binding studies suggest that a significant fraction of the cross bridges may be attached to the thin filament even during relaxation (Chalovich et al., 1981; Wagner & Giniger, 1981; Chalovich & Eisenberg, 1982; Inoue & Tonomura, 1982).

Regulated actin, F-actin plus troponin–tropomyosin, is an in vitro model of the thin filament. It activates the MgATPase of myosin in the presence but not in the absence of calcium (Weber & Murray, 1973). Similar calcium sensitivities are found for the regulated actin-activated ATPases of heavy meromyosin (HMM),[1] a two-headed proteolytic fragment of

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myosin, and subfragment 1 (S1), a single myosin head. Chalovich et al. (1981) used changes in turbidity to measure the affinity of S1 for regulated actin in the presence of MgATP. They found that while removal of calcium inhibited the regulated actin-activated ATPase 20-fold, there was only a slight decrease in acto-S1 affinity. Wagner & Giniger (1981) and Chalovich & Eisenberg (1981, 1982) have confirmed this surprising result using a preparative ultracentrifuge to separate free S1 from that bound to regulated actin. With this technique, the affinity of S1 for regulated actin in the presence of MgATP was found to be independent of calcium. These binding studies indicate that removal of calcium inhibits the regulated actin-activated ATPase of S1 not by preventing binding but rather by inhibiting a subsequent step in the ATPase cycle. However, the binding of HMM to regulated actin in the presence of ATP was partially inhibited by removal of calcium (Wagner & Giniger, 1981); i.e., both binding and a subsequent step were inhibited by troponin–tropomyosin in the absence of calcium. Unfortunately, as it was not possible to measure accurately the binding of HMM to regulated actin in the absence of calcium, we could only estimate that removal of calcium reduced the affinity of HMM for regulated actin at least 4-fold.

In this paper, the calcium-sensitive binding of HMM to regulated actin is more thoroughly investigated. When a slightly lower ionic strength, μ = 18 mM instead of 25 mM, and better HMM preparations are used, it is possible to show that the binding of approximately one-third of the HMM is independent of calcium and that two-thirds of the HMM binds much more weakly in the absence of calcium. A large fraction of the calcium-insensitive HMM results from degradation of the 19 000-dalton light chain, LC2.

The effects of LC2 removal, phosphorylation, and proteolytic degradation on the calcium-sensitive binding of HMM to regulated actin are examined. The interaction of single-headed heavy meromyosin, sHMM, with regulated actin is also investigated. These experiments demonstrate that for calcium-sensitive binding, LC2 must be intact and the head must be attached to the rod portion of HMM, subfragment 2 (S2). The effect of calcium binding to LC2, as opposed to troponin, on the binding of HMM to regulated actin is also examined.

Materials and Methods

Myosin was isolated from rabbit back and white hind leg muscles and actin from the acetone powder (Wagner & Weeds, 1977). Regulated actin was prepared as described previously (Wagner et al., 1979) except equal weights of F-actin and troponin–tropomyosin were mixed. LC2 was isolated from a mixed light chain preparation (Wagner & Weeds, 1977) by precipitation in 18% ethanol (Perrie et al., 1973). HMM was prepared by digesting myosin in 0.6 M NaCl, 2 mM $MgCl_2$, and 20 mM sodium phosphate, pH 7 at 25 °C, for 3 min with 0.025 mg/mL α-chymotrypsin (Weeds & Pope, 1977). After dialysis against 20 mM NaCl, 10^{-4} M DTT, and 10 mM imidazole, pH 7 at 4 °C, and centrifugation to remove undigested myosin and light meromyosin, the HMM was purified by gel filtration on a 2.5 × 100 cm column of Pharmacia S300 equilibrated with 150 mM NaCl, 10^{-4} M DTT, 1 mM NaN_3, 1 mM $MgCl_2$, and 10 mM imidazole, pH 7 at 4 °C.

Protein concentrations were estimated by using the following values for $A^{1\%}_{280}$ and molecular weight: HMM, 6.5 cm^{-1} and 350 000; sHMM, 6.0 cm^{-1} and 250 000; LC2, 6.5 cm^{-1} and 19 000; regulated actin 8.75 cm^{-1} and 63 000 (actin plus 1/7 troponin/tropomyosin).

HMM was depleted of LC2 in a manner similar to that used for myosin (Wikman-Coffelt et al., 1979). HMM, 0.5 mg/mL, was incubated in 0.6 M KCl, 1 mM EDTA, 10 mM ATP, and 100 mM Tris, pH 7.5, for 10 min at 37 °C. It was then added to an equal volume of cold saturated $(NH_4)_2SO_4$, pH 6.5. After 5 min at 0 °C, the solution was centrifuged at 30000g for 20 min. HMM was in the precipitate, while most of the dissociated LC2 remained in the supernatant. All of the following procedures were performed at 4 °C. The pellets were dissolved and dialyzed overnight against 25 mM KCl, 10^{-4} M DTT, 1 mM NaN_3, and 10 mM imidazole, pH 7. This HMM was fractionated on an ADP affinity column (Lamed et al., 1973) equilibrated in 25 mM KCl, 10^{-4} M DTT, 1 mM NaN_3, and 10 mM imidazole, pH 7. For 45 mg of HMM a 2.5 × 30 cm column was used. Approximately 75% of the HMM was eluted as a single peak by a 25–200 mM KCl gradient. The remaining HMM was washed off by 500 mM KCl. The amount of LC2 removed was variable, and the HMM in the first peak was 25–40% depleted of LC2. K^+-EDTA-, Ca^{2+}-, and F-actin-activated ATPases of this HMM were respectively 12, 2, and 7.6 s^{-1} with a K_m (the concentration of actin required to achieve $^1/_2$ V_{max}) of 14 μM. For control HMM, these values were 12, 1.7, and 7.9 s^{-1} with a K_m of 14 μM. The K^+-EDTA ATPase and Ca^{2+}ATPase activities were measured as in Wagner & Giniger (1981), and the actin-activated ATPase activity was measured as in Wagner & Weeds (1979). The HMM which eluted in 500 mM KCl had about half the normal amount of LC2, but the ATPase activities were 30% less than those of control HMM.

LC2 was added back to the depleted HMM as follows. After the 10-min incubation at 37 °C, $MgCl_2$ was added to 14 mM and then isolated LC2 added to 15 μM (a 10-fold molar excess over HMM). After an additional 5 min at 37 °C, the solution was placed on ice for 30 min and then precipitated in 50% saturated $(NH_4)_2SO_4$. This HMM was then fractionated on an ADP affinity column. Free LC2 did not bind to the ADP affinity column. HMM eluting in the 25–200 mM KCl gradient had a full complement of LC2 and normal ATPase activities. If equimolar rather than excess LC2 was added, the LC2 content of the resulting HMM was 85% of control HMM.

sHMM was prepared by digesting HMM with papain in $MgCl_2$ to preserve LC2 (Margossian et al., 1975). sHMM was purified by gel filtration through two serial 2.5 × 100 cm S300 columns, fractionation on an ADP affinity column to separate single- from doubled-headed species, and finally refiltration on a single S300 column. Details of this isolation procedure will be presented elsewhere (R. Mendelson and P. Wagner, unpublished results). When electrophoresed under nondenaturing conditions (Weeds & Pope, 1977), this sHMM gave a single band which migrated between those of HMM and S1. When electrophoresed in $NaDodSO_4$ (Laemmli, 1970), sHMM gave bands with mobilities corresponding to the HMM heavy chain, S2, and the three light chains. There was no band corresponding to the S1 heavy chain. The ATPase activities of sHMM on a per head basis were comparable to those of HMM.

[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; HMM, heavy meromyosin; LC1, light chain 1, M_r 21 000; LC2, light chain 2, M_r 19 000; LC3, light chain 3, M_r 16 500; S1, subfragment 1; S2, subfragment 2; sHMM, single-headed heavy meromyosin; ATPase, adenosinetriphosphatase.

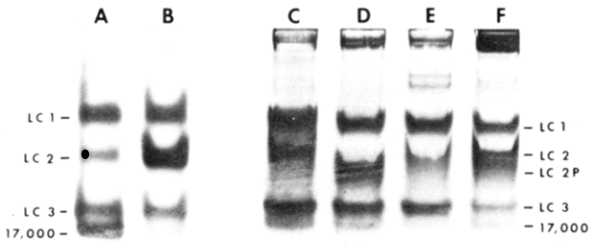

FIGURE 1: Polyacrylamide gel electrophoresis in 8 M urea. (A) HMM isolated from a long chymotryptic digestion of myosin; (B and C) HMM isolated from a short chymotryptic digestion of myosin; (D) phosphorylated HMM; (E) HMM depleted of LC2 by incubation at 37 °C in EDTA and ATP; (F) this HMM was first depleted of LC2 by incubation at 37 °C in EDTA and ATP and then had LC2 added back in $MgCl_2$.

HMM, 4 mg/mL, was phosphorylated by incubation for 20 min at 20 °C in a solution containing 10^{-6} M calmodulin, 5×10^{-8} M myosin light chain kinase, 10 mM $MgCl_2$, 5 mM ATP, 10^{-4} M $CaCl_2$, 50 mM KCl, and 50 mM Tris, pH 7.5 (Cooke et al., 1982). The phosphorylated HMM was stored on ice until used that same day in the binding assays. Light chain kinase and calmodulin were gifts from Dr. Roger Cooke.

Regulated actins at concentrations of 1.5, 3.0, and 6.0 mg/mL were cross-linked with 0.1% glutaraldehyde in the presence and absence of calcium (Mikawa, 1979). After the cross-linking reaction was terminated with KSCN, the fixed actins were dialyzed overnight at 4 °C against 1.25 mM $MgCl_2$, 10^{-4} M DTT, and 6.25 mM imidazole, pH 7. These cross-linked actins were used to activate the MgATPase of HMM under the following conditions: 2 mM ATP, 4 mM $MgCl_2$, and 10 mM imidazole, pH 7 at 20 °C, plus either 10^{-4} M $CaCl_2$ or 2×10^{-4} M EGTA. The assay was performed as in Wagner & Weeds (1979).

The various HMM preparations were examined by gel electrophoresis in $NaDodSO_4$ (Laemmli, 1970) and in 8 M urea (Perrie et al., 1973). These gels were stained with Coomassie Brilliant Blue, and the light chain composition was determined with a scanning gel densitometer. When myosin is digested by chymotrypsin in high salt and divalent cations, some of the LC2 is degraded to a 17000-dalton fragment which comigrates with light chain-3, LC3, when electrophoresed in $NaDodSO_4$. In urea gels, this fragment migrates ahead of LC3 (Figure 1). Electrophoresis in 8 M urea was also used to determine the extent of LC2 phosphorylation.

The extent of HMM binding to regulated actin in the presence of MgATP was determined by using a preparative ultracentrifuge to separate free HMM from that bound to actin (Wagner & Giniger, 1981). The conditions used were 4 mM $MgCl_2$, 2 mM ATP, 10^{-4} M DTT, 0.1 mg/mL bovine serum albumin, and either 10^{-4} M $CaCl_2$ and 10 mM imidazole, pH 7 at 20 °C, or 5×10^{-4} M EGTA and 5 mM imidazole. The ionic strengths of these solutions were calculated to be approximately 18 mM. The HMM was 1.5×10^{-7} M, and the regulated actin varied from 10 to 200 μM. The 1-mL samples were centrifuged for 20 min at 160000g, the supernatants removed, and the NH_4^+-EDTA ATPase activities of 0.5-mL aliquots measured to obtain the concentration of free HMM. When centrifuged in the absence of actin, 90% of the HMM remained in the supernatant. This value was used as a reference in estimating the fraction of HMM bound to regulated actin. The concentrations of HMM in the supernatants were corrected for the small amount of HMM, less than 5%, which did not bind to the regulated actin in the absence of ATP. The concentration of ATP remaining after centrifugation was always greater than 0.5 mM and usually greater than 1 mM.

The conditions for the NH_4^+-EDTA ATPase were 0.44 M NH_4Cl, 39 mM EDTA, 28 mM Tris, and 5 mM ATP, pH 8 at 25 °C. Aliquots were removed after 3 and 13 min and the amounts of inorganic phosphate determined (Rockstein & Herron, 1951). The rate of ATP hydrolysis was linearly dependent on [HMM] in the concentration range used in these experiments. There was no inhibition of this activity by the actin which did not sediment during the centrifugation. Use of the NH_4^+-EDTA ATPase activity to determine the concentration of free HMM is based on a similar assay used by Chalovich & Eisenberg (1981, 1982) to determine the binding of S1 to regulated actin.

The binding of HMM to regulated actin was analyzed as follows:

$$K_a = \frac{[\text{acto·HMM}]}{[\text{HMM}_{free}][\text{actin}_{free}]} \quad (1)$$

$$b = \frac{[\text{acto·HMM}]}{[\text{HMM}_{total}]} \quad (2)$$

It is not possible to distinguish with this binding assay between HMM bound by two heads and HMM bound by only one head. The measured value for [acto·HMM], therefore, includes both types of binding. Since the regulated actin concentration is usually at least 100 times greater than the HMM concentration, competition for actin binding sites is minimal. Only a fraction (n) of the total HMM appears to bind to regulated actin under the conditions used in these assays.

$$[\text{acto·HMM}] + [\text{HMM}_{free}] = n[\text{HMM}_{total}] \quad (3)$$

The total regulated actin concentration is used as $[\text{actin}_{free}]$. Substituting eq 2 and 3 into eq 1 gives

$$nK_a - bK_a = b/[\text{actin}] \quad (4)$$

This is similar to the Scatchard equation. The data were plotted as b vs. $b/[\text{actin}]$ and analyzed by linear regression. The slope is equal to $-K_a$ and the x intercept to n.

The binding of HMM to regulated actin was also monitored by measuring turbidity changes, transmission at 340 nm, in a stopped-flow spectrophotometer. One syringe contained 10 μM HMM, 85 μM regulated actin, 2 mM $MgCl_2$, 10^{-4} M DTT, 20 mM imidazole, and either 10^{-4} M $CaCl_2$ or 2×10^{-4} M EGTA. When the first syringe contained $CaCl_2$, the other syringe contained 6 mM $MgCl_2$, 4 mM ATP, and either 10^{-4} M $CaCl_2$ or 1 mM EGTA. When the first syringe contained EGTA, the other syringe contained 6 mM $MgCl_2$, 4 mM ATP, and either 4×10^{-4} M $CaCl_2$ or 2×10^{-4} M EGTA. All solutions were pH 7 at 20 °C.

Results

HMM Binding. The binding of HMM to regulated actin is shown in Figure 2. (This and all other binding experiments described in this paper were performed in the presence of MgATP.) In the presence of calcium, approximately 90% of the HMM bound with a K_a of 2×10^4 M^{-1}. For other HMM preparations, values for K_a as high as 4×10^4 M^{-1} were obtained. Removal of calcium inhibited the binding of HMM to regulated actin. About one-third of the HMM bound with an affinity similar to that obtained in the presence of calcium (Table I). There was slightly more HMM bound at the highest regulated actin concentration than could be accounted for by the calcium-insensitive HMM; in Figure 2 the amount of HMM bound at 180 μM regulated actin lies above the line

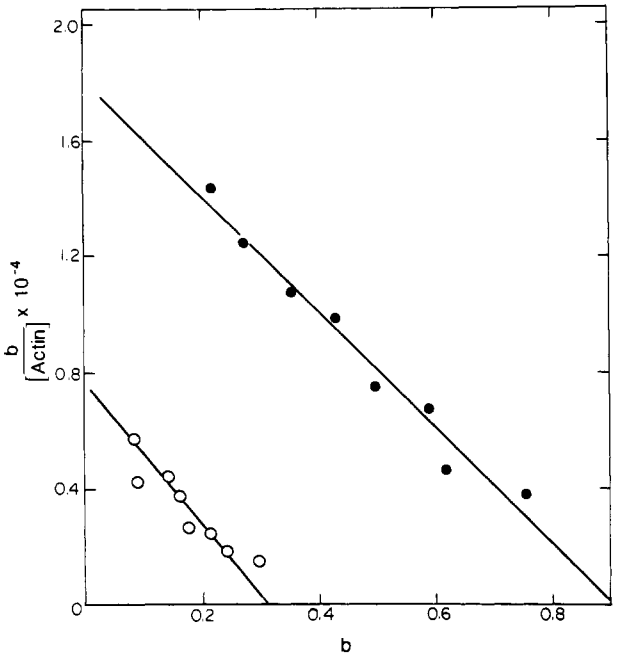

FIGURE 2: Binding of HMM to regulated actin in the presence of MgATP. Conditions for binding in calcium (●) were 10 mM imidazole, 2 mM ATP, 4 mM $MgCl_2$, 10^{-4} M $CaCl_2$, 10^{-4} M DTT, and 0.1 mg/mL bovine serum albumin, pH 7.0 at 20 °C. Conditions for binding in the absence of calcium (○) were 5 mM imidazole, 2 mM ATP, 4 mM $MgCl_2$, 5×10^{-4} M EGTA, 10^{-4} M DTT, and 0.1 mg/mL bovine serum albumin, pH 7.0 at 20 °C. *b* is the fraction of HMM bound to the regulated actin.

Table I: Summary of the Binding of HMM to Regulated Actin in the Presence of MgATP

	calcium		EGTA	
	n^a	$K_a \times 10^{-4}$ (M^{-1})	n^a	$K_a \times 10^{-4}$ (M^{-1})
HMM	0.91	2.0	0.31	2.5
sHMM	0.80	1.1	ND[b]	ND[b]
HMM – LC2[c]	0.80	3.7	0.73	3.3
HMM + LC2[d]	0.91	2.6	0.37	5.2
HMM with 60% degraded LC2[e]	0.91	4.0	0.74	2.6
HMM, phosphorylated LC2[f]	0.93	3.3	0.31	5.0

[a] Values for *n* and K_a were obtained from linear regressions of plots of *b* vs. *b*/[actin]. [b] There was too little binding even at the highest regulated actin concentrations for meaningful values of *n* and K_a to be calculated. [c] This HMM was 40% depleted of LC2 by incubation at 37 °C in EDTA and ATP. [d] This HMM was first depleted of LC2 by incubation at 37 °C in EDTA and ATP and then had LC2 added back in $MgCl_2$. It contained 100% LC2. [e] HMM isolated from a high chymotryptic digestion of myosin. It contained 40% intact LC2. [f] All of the 85–90% intact LC2 was phosphorylated by myosin light chain kinase.

drawn for a single binding population. This was observed for three different HMM preparations and indicates that the rest of the HMM may be able to bind to regulated actin in the absence of calcium but with a greatly reduced affinity.

sHMM Binding. The binding of HMM to regulated actin is calcium sensitive, while the binding of S1, with or without LC2, is not calcium sensitive (Wagner & Giniger, 1981). This difference in calcium sensitivity could result either from head–head interactions within HMM or from the attachment of these heads to the myosin rod. sHMM contains a single head which is still attached to S2. The binding of sHMM to regulated actin is shown in Figure 3. In the presence of

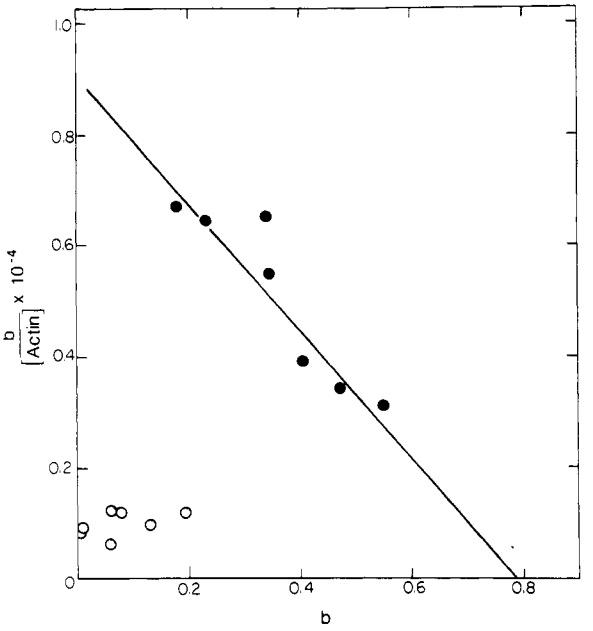

FIGURE 3: Binding of sHMM to regulated actin in the presence of MgATP. The conditions were the same as those given in Figure 2; (●) in calcium; (○) in EGTA.

calcium, 80% of the sHMM bound with a K_a of 1.1×10^4 M^{-1}, about half as strongly as HMM under the same conditions. In the absence of calcium, there was not enough sHMM binding to make a reasonable estimate of K_a. Between 30 and 120 μM the fraction of sHMM bound fluctuated between 0 and 10%. Only at the highest regulated actin concentrations did the binding increase to around 20%. At a slightly higher ionic strength, μ = 25 mM, sHMM bound to regulated actin in the presence of calcium with a K_a of 8.3×10^3 M^{-1}. In the absence of calcium, less than 5% of the sHMM was bound at 170 μM regulated actin. Similar results were obtained with two other sHMM preparations.

LC2 Removal. Removal of LC2 by incubation of HMM at 37 °C in EDTA and ATP followed by fractionation on an ADP affinity column yielded HMM with full ATPase activities and substantial depletion of LC2 (Figure 1). This method was used as we had difficulty obtaining complete recovery of ATPase activity when LC2 was removed by treatment with 5,5′-dithiobis(2-nitrobenzoic acid).

The binding of HMM containing 60% intact LC2 to regulated actin is shown in Figure 4A. In the presence of calcium, 80% of this HMM bound, K_a of 3.7×10^4 M^{-1}. In the absence of calcium, 73% bound with a similar affinity; see Table I. Thus, only about 10% of this LC2-depleted HMM showed calcium-sensitive binding. LC2 was added back to this depleted HMM (see Materials and Methods) (Figure 1). As shown in Figure 4B, this addition of LC2 restored the fraction of HMM with calcium-sensitive binding to about 60%, almost as much as in control HMM (Table I). This shows that most of the loss of calcium sensitivity is due to LC2 removal and not to the incubation of HMM at 37 °C.

Digestion of LC2. HMM prepared from myosin as described under Materials and Methods contains 85–90% intact LC2; the remaining 10–15% has been digested to a 17 000-dalton fragment which remains bound to the HMM. Increasing the chymotrypsin to 0.1 mg/mL and the time of digestion to 10 min resulted in a HMM preparation in which 60% of LC2 had been degraded to the 17 000 dalton fragment (Figure 1). The heavy chains, LC1, and LC3 appeared to be the same in HMM prepared by either the mild or the extensive digestion.

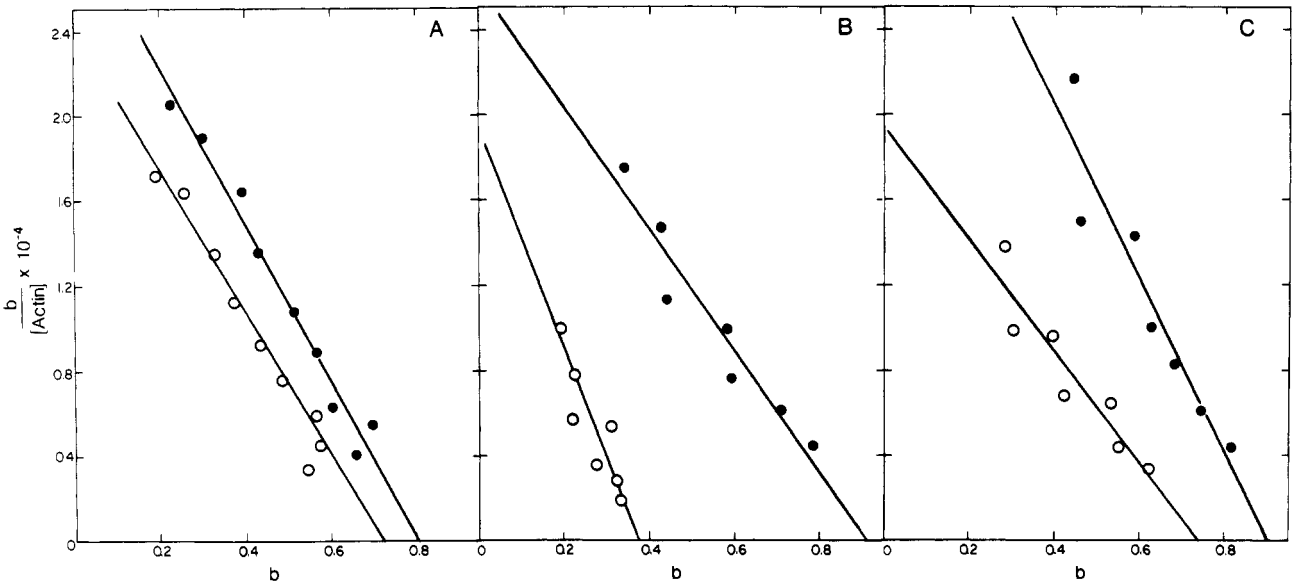

FIGURE 4: Effect of LC2 on the binding of HMM to regulated actin in the presence of MgATP. (A) HMM was depleted of LC2 by incubation at 37 °C in EDTA and ATP. There were 1.2 mole of LC2/mol of HMM. (B) LC2 was added back to HMM which had been depleted of LC2 by incubation at 37 °C in EDTA and ATP. There were 2 mol of LC2/mol of HMM. (C) HMM was isolated from an extensive digestion of myosin. It contained 40% intact LC2; the other 60% was present as a 17 000-dalton fragment. Conditions were the same as in Figure 2; (●) in calcium; (○) in EGTA.

Digestion of LC2 to the 17 000-dalton fragment has little effect on the binding of HMM to regulated actin in the presence of calcium (Table I). However, as shown in Figure 4C, the fraction of HMM with calcium-sensitive binding is greatly reduced; 74% bound with an affinity similar to that observed in the presence of calcium (Table I).

Phosphorylation of LC2. HMM as routinely prepared contains no phosphorylated LC2. All of the intact LC2 can be phosphorylated when HMM is incubated with light chain kinase and calmodulin (see Materials and Methods) (Figure 1). Subsequent incubations under the conditions used in the binding assays do not alter the level of phosphorylation as observed by gel electrophoresis in 8 M urea. Phosphorylation has no discernible effect on the calcium-sensitive binding of HMM to regulated actin (Table I).

Cross-Linked-Regulated Actin. The calcium-sensitive binding of HMM to regulated actin could result solely from calcium binding to troponin, or calcium binding to LC2 could also influence this interaction. Under the conditions used in these binding assays, magnesium will be bound to LC2 when EGTA is present, but calcium will replace most of the magnesium on LC2 when calcium is added in place of the EGTA. To help resolve whether this calcium binding to LC2 is responsible for the observed calcium sensitivity, regulated actin was cross-linked with 0.1% glutaraldehyde both in the presence of calcium to give regulated actin fixed in an "active" conformation and in the presence of EGTA to give regulated actin fixed in an "inhibited" conformation (Mikawa, 1979).

The MgATPase of HMM was measured at varying concentrations of fixed-active regulated actin (Figure 5). The maximum activity and K_m were the same in the presence and absence of calcium, 3.9 s^{-1} and 27 μM. When control regulated actin was used in the presence of calcium, V_{max} and K_m were 3.9 s^{-1} and 25 μM, and in the absence of calcium, the ATPase was 95% inhibited. The similarity of V_{max} and K_m for the fixed-active regulated actin and for control regulated actin in calcium suggests that fixed actin accurately mimics the native conformation. The binding of HMM to fixed-active regulated actin was calcium insensitive, 85% bound with a K_a of 7.1 × 10^4 M^{-1} both in calcium and in EGTA. Both the

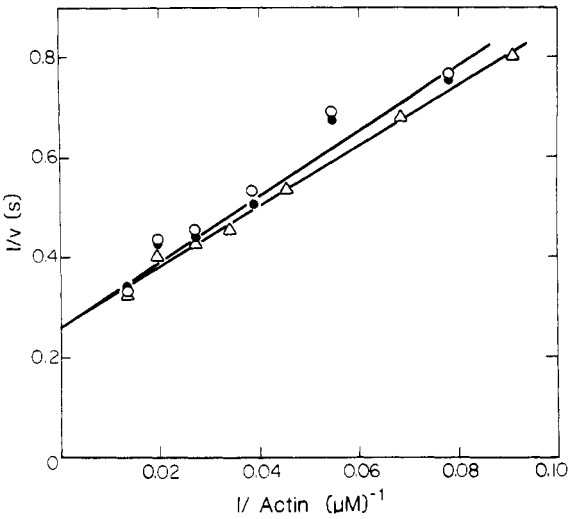

FIGURE 5: Double-reciprocal plot of the regulated actin activated ATPase of HMM. Assay conditions were 10 mM imidazole, 2 mM ATP, 4 mM $MgCl_2$, 10^{-4} M DTT, and 3 × 10^{-7} M HMM, pH 7.0 at 20 °C. (△) Control regulated actin assayed in 10^{-4} M $CaCl_2$; (●, ○) regulated actin cross-linked in the presence of calcium; (●) assayed in 10^{-4} M $CaCl_2$; (○) assayed in 2 × 10^{-4} M EGTA.

binding and ATPase measurements indicate that the affinity of HMM for regulated actin in the active state is not affected by calcium binding to LC2.

When regulated actin was cross-linked with glutaraldehyde in EGTA, most, but not all, was fixed in an inhibited state. This was true whether the regulated actin was cross-linked at 1.5, 3.0, or 6.0 mg/mL. In both calcium and EGTA, these fixed regulated actins activated the ATPase of HMM to a rate about one-fourth that obtained with the same concentration of fixed-active regulated actin. In calcium and in EGTA, 40% of HMM bound to this fixed-inhibited regulated actin with a K_a of 7.1 × 10^4 M^{-1}. There was no increase in HMM binding to this inhibited actin when calcium was bound to LC2.

Stopped-Flow Measurements of Turbidity Changes. The rate constants for the dissociation of calcium and magnesium from LC2 have half-lives of 1.5 and 12.5 s, respectively (Bagshaw & Reed, 1977). The rates of association and dis-

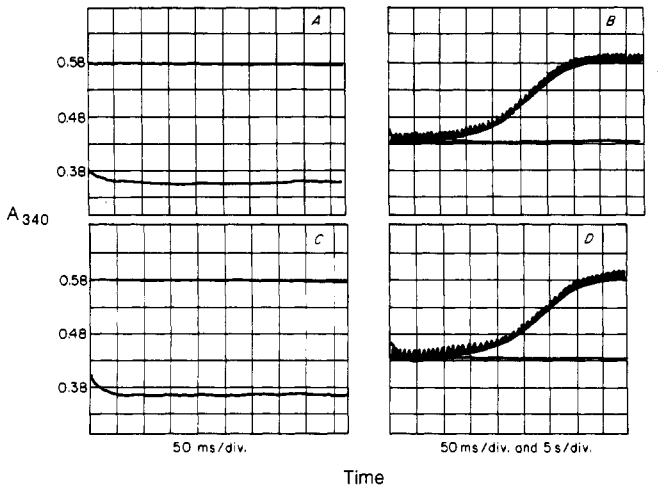

FIGURE 6: Rate of change in the binding of HMM to regulated actin. In (A) and (B), one syringe contained 10 μM HMM, 85 μM regulated actin, 2 mM $MgCl_2$, 10^{-4} M DTT, 20 mM imidazole, and 2×10^{-4} M EGTA. The other syringe contained 4 mM ATP, 6 mM $MgCl_2$, and either (A) 2×10^{-4} M EGTA or (B) 4×10^{-4} M $CaCl_2$. In (C) and (D), one syringe contained 10 μM HMM, 85 μM regulated actin, 2 mM $MgCl_2$, 10^{-4} M DTT, 20 mM imidazole, and 10^{-4} M $CaCl_2$. The other syringe contained 4 mM ATP, 6 mM $MgCl_2$, and either (C) 1 mM EGTA or (D) 10^{-4} M $CaCl_2$. All solutions were pH 7.0 at 20 °C. The bottom traces in (A) and (C) show the absorbance change immediately after mixing, and the top traces show absorbance after all of the ATP had been hydrolyzed, about 10 min later. In (B) and (D), one trace was recorded at 5 ms/division and the other at 5 s/division. The absorbance increases after 25–30 s occurred when all the ATP had been hydrolyzed.

sociation of calcium with troponin are much faster (Johnson et al., 1980). By rapid mixing in a stopped-flow apparatus, it is possible to have transiently a solution in which calcium is bound to troponin but not to LC2, or conversely calcium bound to LC2 but not to troponin. These solutions can be used to check whether calcium binding to LC2 affects the affinity of HMM for regulated actin.

The binding of HMM to regulated actin was followed by measuring turbidity changes; transmission at 340 nm was used to measure turbidity (Chalovich et al., 1981). The greater the fraction of HMM bound to regulated actin the greater the turbidity. When HMM plus regulated actin was mixed in a stopped-flow spectrophotometer with a buffer containing no MgATP, the turbidity did not become constant until after about 50 ms. This reflects the rather slow mixing of the viscous acto-HMM solution. Fortunately, the reactions of interest are slow enough that this delay was not a problem.

When HMM plus regulated actin in EGTA was mixed with MgATP plus EGTA, the turbidity fell rapidly, attaining a constant value, 0.36, after about 50 ms (Figure 6A). It remained at this level until the ATP was hydrolyzed, about 10 min. The top trace in Figure 6A shows the turbidity (about 0.58) after all of the HMM had rebound to the regulated actin. As the initial steady-state turbidity was higher than that of completely dissociated HMM, 0.31, some of the HMM was bound to the regulated actin in the absence of calcium. Complete dissociation of HMM from regulated actin was obtained by including 1.2 M KCl in the syringe containing the MgATP and EGTA.

The same solution of HMM plus regulated actin in EGTA was mixed with MgATP and enough $CaCl_2$ to activate the regulated actin activated ATPase (Figure 6B). After the first 50 ms, the turbidity reached a steady-state value of 0.43. It remained reasonably constant until all the ATP was hydrolyzed (about 25 s); it then rose as all the HMM became bound. In this experiment, immediately after mixing, calcium will be bound to troponin, but magnesium will still be bound to LC2. The rate of calcium binding to LC2 will be limited by the dissociation of magnesium, $t_{1/2}$ = 12.5 s. As there was no change in turbidity during this time, the affinity of HMM for regulated actin (with calcium bound to troponin) is the same whether magnesium or calcium is bound to LC2. If the affinity of HMM for regulated actin was totally dependent on calcium binding to LC2, the turbidities immediately after mixing would have been the same in Figure 6A,B.

In a second set of experiments, HMM plus regulated actin in calcium was mixed in a stopped-flow spectrophotometer with MgATP plus either calcium or enough EGTA to inhibit the regulated actin activated ATPase. When mixed with MgATP plus $CaCl_2$, the turbidity reached a steady-state level within about 50 ms where it remained until the ATP had been hydrolyzed (Figure 6D). When mixed with MgATP plus EGTA (Figure 6C), the turbidity immediately after mixing was lower than that observed in calcium, and it remained at this level until all the ATP was hydrolyzed, about 10 min. The steady-state turbidity was the same as that obtained when HMM plus regulated actin in EGTA was mixed with MgATP plus EGTA (Figure 6a). In this experiment, Figure 6C, immediately after mixing, calcium was still bound to LC2 but not to troponin. The rate constant for the dissociation of calcium from LC2 has a $t_{1/2}$ of 1.5 s. As there was no change in turbidity with this rate constant, the affinity of HMM for regulated actin (without calcium bound to troponin) appears to be the same whether calcium or magnesium is bound to LC2.

Discussion

In a previous paper (Wagner & Giniger, 1981) we reported that the binding of HMM to regulated actin, unlike that of S1, was dependent on the calcium ion concentration. It was not possible to tell from those experiments whether removal of calcium resulted only in a 4-fold decrease in affinity of all the HMM, or whether there were two populations of HMM with widely different affinities for regulated actin in the absence of calcium. The following changes in experimental design were made to resolve this ambiguity. HMM was filtered through a column to remove a 5% S1 contamination. The affinity of HMM for regulated actin was increased by lowering the ionic strength from 25 to 18 mM. Previously, radiolabeled LC1 was exchanged into HMM and this radioactivity used to determine the concentration of HMM in the supernatant after centrifugation. While this exchange had minimal effects on the ATPase activities of HMM, it did require incubating HMM under dissociating conditions. Use of the NH_4^+-EDTA ATPase to measure the HMM concentration eliminated these manipulations and allowed for fresher HMM to be used. (K_a for the binding of HMM to regulated actin in the presence of calcium was the same whether the NH_4^+-EDTA ATPase or radiolabeled LC1 was used to determine the concentration of free HMM.) HMM was used within 5 days of myosin digestion. Finally, the HMM was isolated from a 25% chymotryptic digestion of myosin instead of from the 40% digestion used previously. This gave HMM with a higher content of intact LC2. These modifications made it possible to analyze more fully the calcium-sensitive binding of HMM to regulated actin. The analysis is still limited by the inability to use concentrations of regulated actin higher than about 200 μM in the binding assays. Regulated actin above this concentration is very viscous and, in our hands, impossible to mix reproducibly.

The affinity of approximately one-third of the HMM for regulated actin, in the presence of MgATP, is independent of

calcium concentration (Table I). The binding of the rest of the HMM is regulated by calcium. In the absence of calcium, this latter HMM binds very weakly, if at all, to regulated actin. In plots of b vs. $b/[\text{actin}]$ for the binding of HMM in the absence of calcium, the data obtained at the highest regulated actin concentrations tend to lie above the line calculated assuming only one type of HMM binding. This indicates that in addition to the one-third of the HMM with calcium-insensitive binding, there is some additional HMM binding. There was too little binding of the calcium-sensitive HMM to regulated actin in the absence of calcium to allow for an accurate determination of this association constant. It was estimated by trying various values and calculating the amount of HMM that would be bound at different regulated actin concentrations. While 10- and 15-fold reductions in affinity predicted more binding than was observed, a 20-fold reduction gave values that were consistent with the small amounts of additional binding observed at the highest regulated actin concentrations. However, with the difficulty of obtaining precise data at low levels of saturation and the lack of data at high saturations, it is possible that there is only a 10-fold reduction in affinity.

The binding of sHMM to regulated actin is also calcium sensitive. In the absence of calcium the data were too scattered to allow one to make reliable estimates of either n or K_a. Nor was it possible to tell whether a fraction of the sHMM was binding independent of calcium. The reason for the scatter at low levels of saturation is that a small percentage error in the measurement of free sHMM causes a large percentage error in the value calculated for bound sHMM. If one assumes that 80% of the sHMM (the fraction which binds in calcium) binds with a single affinity, an average value of 1.1×10^3 M^{-1} is obtained for K_a, approximately 10-fold weaker than in calcium. If a small fraction of the sHMM has calcium-insensitive binding, the affinity of the rest would then be considerably less than 1.1×10^3 M^{-1}.

In the presence of calcium, sHMM binds only 2-fold more weakly than HMM. This suggests that under these conditions the second head of HMM contributes very little to the affinity of HMM for regulated actin. This apparent small contribution by the second head of HMM is also evident in the small differences in the affinities of S1 and HMM for regulated actin (Wagner & Giniger, 1981) and in the K_m values of both the F-actin-activated ATPases (Wagner et al., 1979) and the regulated actin activated ATPases (Wagner & Giniger, 1981) of S1 and HMM. However, both heads of HMM appear to interact with actin under these conditions as the rates of ATP hydrolysis per mole of HMM are approximately twice those of S1 (Wagner et al., 1979). Greene (1981) has found that the contribution made by the second head to the affinity of HMM for actin depends on ionic strength, temperature, and presence of nucleotide.

Degradation of LC2 probably accounts for some of the HMM with calcium-insensitive binding. HMM isolated from a more extensive chymotryptic digestion of myosin contained only 40% intact LC2; the other 60% was degraded to 17 000-dalton fragments. In a random distribution, 84% of the HMM would have contained at least one degraded LC2. The binding of 83% of this HMM to regulated actin was independent of calcium; i.e., 75% bound in EGTA with an affinity similar to that of the 90% which binds in calcium (Table I). If the binding of one head of HMM is made calcium insensitive by LC2 cleavage, the whole HMM will appear to bind independent of calcium as the binding assay does not distinguish HMM bound by one head from that bound by two heads. In HMM prepared by the normal mild chymotryptic digestion, 10–15% of these light chains have been degraded to 17 000-dalton fragments. If randomly distributed, 19–28% of the HMM would contain degraded LC2. Thus, LC2 degradation appears to account for most of the calcium-insensitive HMM in the normal HMM preparations. This digestion of LC2 occurs after a phenylalanine residue 18 amino acids from the N terminus (Weeds & Pope, 1977). There is no evidence on whether this 2000-dalton fragment remains bound to HMM. HMM isolated from even lower chymotryptic digestion did not contain significantly more intact LC2. sHMM contains nearly stoichiometric amounts of intact LC2.

Removal of LC2 by incubation of HMM in EDTA and ATP at 37 °C also resulted in an increase in calcium-insensitive binding of HMM to regulated actin. A larger fraction of this HMM lacked calcium sensitivity than could be accounted for by random LC2 removal. While 60% of LC2 remained, 90% (72/80) of the HMM bound in the presence of EGTA. If the first LC2 is dissociated more readily than the second, 80% of the HMM would contain only one LC2. This would account for most of the calcium-insensitive binding. Readdition of LC2, so that the HMM contained nearly 100% intact LC2, restored most but not all of the calcium sensitivity (Table I). Thus, some of the loss in calcium sensitivity probably results from the lability of this property. Indeed, when HMM was stored on ice for 2 weeks, the fraction with calcium-insensitive binding increased.

Inoue & Tonomura (1982) have reported that under conditions similar to those used in the experiments reported here, there is little difference in the affinity of HMM for regulated actin in the presence and absence of calcium. Their HMM was prepared by a tryptic digestion of myosin. Most of the LC2 in this type of HMM is degraded (Weeds & Pope, 1977), and this degradation probably accounts for the lack of calcium sensitivity.

The divalent cation site on LC2, most likely between residues 37 and 48 (Bagshaw & Kendrick-Jones, 1980), has a higher affinity for calcium than for magnesium. Under relaxing conditions, magnesium is bound to this site. The calcium concentration under contracting conditions is high enough for it to displace a large fraction of the magnesium from LC2. However, the rate of dissociation of magnesium from LC2 and, therefore, the rate of calcium binding are much too slow, $t_{1/2}$ of 12.5 s (Bagshaw & Reed, 1977), for this exchange to have a regulatory role during a single twitch. But it is possible that calcium binding to LC2 may have some regulatory function during prolonged stimulation. Both the equilibrium binding of HMM to cross-linked regulated actins and the rates of change and turbidity after HMM and regulated actin are rapidly mixed with MgATP showed no differences in the affinity of HMM for regulated actin whether calcium or magnesium was bound to LC2. Thus, the calcium-sensitive binding of HMM to regulated actin appears to result solely from calcium binding to troponin. However, the proximity of the divalent cation site on LC2 to the N-terminal 18 amino acids which are required for calcium-sensitive binding suggests that this site may be structurally important. Divalent cation binding, either calcium or magnesium, to isolated LC2 appears to cause a large change in shape (Alexis & Gratzer, 1978). However, divalent cation binding to the isolated 17 000-dalton fragment of LC2 did not cause any large change in shape. This also suggests an interaction between the N-terminal 18 amino acids and the divalent cation site. In addition to binding divalent cations, LC2 is phosphorylated by a myosin light chain specific kinase at serine-13. This phosphorylation appears to

be involved in controlling the rate of ATP hydrolysis during prolonged stimulation (Crow & Kushmerick, 1982; Cooke et al., 1982). While this phosphorylation site is within the N-terminal segment of LC2 which is necessary for the calcium-sensitive binding of HMM to regulated actin, phosphorylation had no obvious effect on this calcium sensitivity (Table I).

The binding of S1, with or without LC2, to regulated actin is calcium insensitive (Wagner & Giniger, 1981). Since we have found sHMM to be calcium sensitive, the loss of calcium sensitivity in S1 results not from the lack of interaction between the two heads of myosin but rather from cleavage of the S1/S2 junction. It is not known whether this results simply from separating S1 from S2 or whether it is the removal of part of the heavy chain that causes this loss of calcium sensitivity. The requirement for both an intact S1/S2 junction and an intact LC2 for this calcium sensitivity suggests that LC2 may bind to this part of the myosin heavy chain. Divalent cation binding to LC2 protects this part of the heavy chain from proteolysis by chymotrypsin (Bagshaw, 1977; Weeds & Pope, 1977). The electron microscope has provided more direct evidence that the analogous light chain of scallop adductor muscle myosin is located in the S1/S2 junction (Vibert & Craig, 1982; Flicker et al., 1981). While it seems likely that LC2 binds to the S1/S2 region, part of it may also extend into S1, as this type of light chain appears to be quite long, about 100 Å (Stafford & Szent-Györgyi, 1978; Alexis & Gratzer, 1978; Hartt & Mendelson, 1979).

While it is not possible, at this time, to describe how LC2 and the S1/S2 junction interact to cause calcium-sensitive binding of HMM to regulated actin, two general explanations seem plausible. Part of LC2, most likely the N terminus, may be close to the actin binding site and physically interact with tropomyosin when it is in the blocking position. [HMM binding to F-actin is not calcium sensitive (Wagner & Giniger, 1981).] The interaction of LC2 with the S1/S2 junction would be necessary for LC2 to be held in the correct position on the myosin head. The other type of explanation is that the S1/S2 junction or a properly positioned LC2 is necessary to produce the correct conformation at the actin binding site but is physically remote from it.

While a number of investigations have shown an influence of LC2, or of calcium binding to LC2, on actin–myosin interactions (Margossian et al., 1975, 1980; Pemrick, 1977; Lehman, 1977; Wikman-Coffelt et al., 1979; Moss et al., 1982; Oda et al., 1980; Srivastava et al., 1980), no consistent explanation has emerged. There is a number of similarities between the calcium-sensitive binding of vertebrate striated muscle HMM to regulated actin and the calcium-sensitive actin-activated ATPase of scallop adductor muscle myosin. This ATPase is regulated by calcium binding to the myosin [reviewed by Kendrick-Jones & Scholey (1981)]. This calcium-sensitive ATPase requires a regulatory light chain (Kendrick-Jones et al., 1976) and an intact S1/S2 junction (Stafford et al., 1979). The divalent cation site which is solely on the regulatory light chain is structural rather than regulatory (Bagshaw & Kendrick-Jones, 1979). There are also similarities with vertebrate smooth muscle myosins. The actin-activated ATPases of these myosins are regulated, at least in part, by phosphorylation of their regulatory light chains. This regulatory mechanism is also operative in smooth muscle HMM (Seidel, 1978; Onishi & Watanabe, 1979) and in single-headed myosin (Nath et al., 1982), but not in S1 even though the regulatory light chain can still be phosphorylated (Nath et al., 1982). Thus, in all three types of muscle regulation, the S1/S2 junction and the regulatory light chains appear to play important and similar roles.

The regulated actin activated ATPase of HMM in the absence of calcium is only about 5% of that in the presence of calcium, but even in our best HMM preparations, one-third of the HMM binds independent of calcium. Therefore, troponin–tropomyosin can inhibit the interaction of HMM with the actin at two different steps in the ATPase cycle, attachment and a step which occurs after binding but prior to product release.

It is difficult to extrapolate the results of these in vitro binding studies to the intact muscle and to decide whether the inhibition of cycling or some subsequent step is the more important in controlling muscle contraction. Relaxed muscles have very low stiffness. As stiffness is believed to be proportional to the number of attached cross bridges, it would appear that very few are attached during relaxation. However, recent measurements of stiffness using very rapid stretches at 5 °C and μ = 18 mM indicate that the cross bridges can be attached to the thin filament without developing force (Schoenberg et al., 1982). On the other hand, with slower stretches, while the contracting fiber was stiff, the relaxed fiber was not. This appears to indicate some difference in the attachment of cross bridges in relaxed and contracting fibers. Thus, it is not clear even under these conditions, let alone at physiological ionic strength, whether inhibition of attachment or cycling is the predominate mechanism of regulation.

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Two New Bifunctional Protein Modification Reagents and Their Application to the Study of Parvalbumin†

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ABSTRACT: 2-[(Trifluoroacetoxy)mercuri]-4-fluorophenol (MFP) and 4-(acetoxymercuri)phenyl azide (MPA) have been prepared and characterized. The sulfhydryl-specific reagents MFP and MPA have been used to study the structure of parvalbumin. The ^{19}F resonance of the derivative of parvalbumin with MFP was studied at 94.6 and 235.2 MHz. At 94.6 MHz, the ^{19}F signal line width is 25 Hz and T_1 = 0.28 s, while at 235.2 MHz, the line width is 35 Hz and T_1 = 0.39 s. T_1 was not affected by substitution of D_2O for H_2O as a solvent. Removal of calcium from the parvalbumin derivative resulted in the upfield shift of the ^{19}F signal and a decrease in T_1 at 94.6 MHz. The pK_a for phenolic titration of the MFP derivative was 10.75 compared with 9.5 for the free reagent. These results are interpreted in terms of lodgment of the aryl group in a cleft in the surface of the parvalbumin. The derivative with MPA was photolyzed and subjected to amino acid analysis. Comparison of the analysis of parvalbumin, parvalbumin–MPA, and the photolyzed product indicated destruction of aspartic acid during the photolysis. Asp-22 is a reasonable candidate for the residue attacked, based on comparison with the published crystal structure of parvalbumin.

Sulfhydryl groups show an affinity for free or nonsubstituted mercuric ions, whence the name mercaptan (mercuri aptum, fitted for mercury) is derived. Dissociation constants for CH_3Hg^+ with RS^- are in the range of 10^9 times lower than those for CH_3Hg^+ with other ligands, making alkyl or aryl mercury reagents, in general, good candidates for selective labeling of sulfhydryl groups in proteins. In this paper, we present the synthesis of two reagents, 2-[(trifluoroacetoxy)-mercuri]-4-fluorophenol (MFP)[1] and 4-(acetoxymercuri)-phenyl azide (MPA), and explore their usefulness in the study of the structure of parvalbumin, a calcium-binding protein from vertebrate muscle. The fluorinated mercuriphenol was designed with the idea of introducing a fluorinated phenol into

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[1] Abbreviations: Me_2SO-d_6, dimethyl-d_6 sulfoxide; IR, infrared; MFP, 2-[(trifluoroacetoxy)mercuri]-4-fluorophenol; MPA, 4-(acetoxymercuri)phenyl azide; NMR, nuclear magnetic resonance; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; TFA, trifluoroacetic acid; GSH, glutathione.

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