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Effect of Skeletal Muscle Myosin Light Chain 2 on the Ca^{2+}-Sensitive Interaction of Myosin and Heavy Meromyosin with Regulated Actin†

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ABSTRACT: A low-speed centrifugation assay has been used to examine the binding of myosin filaments to F-actin and to regulated actin in the presence of MgATP. While the cross-linking of F-actin by myosin was Ca^{2+} insensitive, much less regulated actin was cross-linked by myosin in the absence of Ca^{2+} than in its presence. Removal of the 19 000-dalton, phosphorylatable light chain from myosin resulted in the loss of this Ca^{2+} sensitivity. Readdition of this light chain partially restored the Ca^{2+}-sensitive cross-linking of regulated actin by myosin. Urea gel electrophoresis has been used to distinguish that fraction of heavy meromyosin which contains intact phosphorylatable light chain from that which contains a 17 000-dalton fragment of this light chain. In the absence of Ca^{2+}, heavy meromyosin which contained digested light chain bound to regulated actin in MgATP about 10-fold more tightly than did heavy meromyosin which contained intact light chain. The regulated actin-activated ATPases of heavy meromyosin also showed that cleavage of this light chain causes a substantial increase in the affinity of heavy meromyosin for regulated actin in the absence of Ca^{2+}. Thus, the binding of both myosin and heavy meromyosin to regulated actin is Ca^{2+} sensitive, and this sensitivity is dependent on the phosphorylatable light chain.

In vertebrate striated muscles, contraction is regulated by Ca^{2+} binding to troponin on the thin filament. In the absence of Ca^{2+}, troponin–tropomyosin inhibits the cyclic interaction of actin with myosin. Ca^{2+} binding to troponin causes a shift in the position of tropomyosin on the thin filament (Huxley, 1972; Haselgrove, 1972; Parry & Squire, 1973), allowing for myosin to interact with actin and force to be developed. An in vitro model of the thin filament is regulated actin, F-actin, and troponin–tropomyosin. In the presence but not in the absence of Ca^{2+}, regulated actin activates the MgATPase of myosin, heavy meromyosin (HMM),[1] and myosin subfragment

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1 (S1). As the affinity of S1 for regulated actin in MgATP is only slightly affected by the Ca^{2+} concentration (Chalovich et al., 1981; Wagner & Giniger, 1981; Chalovich & Eisenberg, 1982; Inoue & Tonomura, 1982), troponin–tropomyosin does not inhibit the actin-activated ATPase of S1 in the absence of Ca^{2+} by preventing S1 from binding to actin, but rather some subsequent step in the ATPase cycle is inhibited. However, the affinities of HMM and single-headed HMM for regulated actin in MgATP are Ca^{2+} dependent (Wagner & Giniger, 1981; Wagner & Stone, 1983a). This Ca^{2+} sensitivity results from Ca^{2+} binding to troponin and not to the phosphorylatable light chain of myosin, LC2 (Wagner & Stone, 1983a). Thus, both binding of HMM to regulated actin in MgATP and some subsequent step in the ATPase cycle are inhibited by troponin–tropomyosin in the absence of Ca^{2+}.

HMM made by a low chymotryptic digestion of myosin contains mostly intact LC2, but 10–15% of these light chains are present as 17 000-dalton fragments. In the absence of Ca^{2+}, while one-third of this HMM bound to regulated actin in MgATP with the same affinity as in Ca^{2+}, the rest appeared to bind much more weakly. The Ca^{2+}-insensitive HMM was thought to be that fraction which contained digested LC2, as HMM depleted of LC2 and HMM which contained mostly digested LC2 bound to regulated actin almost as well in EGTA as in Ca^{2+} (Wagner & Stone, 1983a). Chalovich & Eisenberg (1984) have also measured the binding of HMM to regulated actin in the presence of MgATP. However, these authors found no evidence for biphasic binding of HMM to regulated actin and observed only a 3-fold difference in the affinity of HMM for regulated actin in Ca^{2+} and in EGTA.

In this paper, urea gel electrophoresis is used to provide direct evidence that HMM which contains digested LC2 has a significantly higher affinity for regulated actin in the presence of MgATP and EGTA than does HMM which contains intact LC2. The regulated actin-activated ATPases of HMM which contains mostly digested LC2 are compared to those of HMM which contains mostly intact LC2 to see if this change in affinity affects K_{app}, the actin concentration required to achieve half the maximum ATPase rate. A low-speed centrifugation assay is used to demonstrate the Ca^{2+}-sensitive binding of myosin filaments to regulated actin in the presence of MgATP. The effect of LC2 removal on this interaction is also determined.

Materials and Methods

Myosin was isolated from rabbit back and white hind leg muscles, and actin and troponin–tropomyosin were prepared from the acetone powder of these muscles (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). Myosin was digested at 15 mg/mL in 0.6 M NaCl, 2 mM $MgCl_2$, and 20 mM sodium phosphate, pH 7 at 25 °C, either with 0.025 mg/mL α-chymotrypsin for 3 min to give HMM with mostly intact LC2 or with 0.1 mg/mL α-chymotrypsin for 10 min to give HMM with mostly digested LC2 (Weeds & Pope, 1977; Wagner & Stone, 1983a). HMM was purified by gel filtration on Sephacryl S300 (Pharmacia).

Myosin was partially depleted of LC2 by incubation at 37 °C in EDTA and ATP (Wikman-Coffelt et al., 1979) except 1 mM DTT was included. A 5-min incubation was used as myosin incubated for 10 min at 37 °C gave large aggregates when dialyzed into 150 mM KCl. Reducing the incubation time to 5 min eliminated this problem. LC2 was added back to this myosin as described previously (Wagner & Stone, 1983b). The $(NH_4)_2SO_4$-precipitated myosins were dissolved in 0.6 M KCl and 1 mM DTT and dialyzed overnight against 0.15 M KCl, 10 mM imidazole, and 10^{-4} M DTT, pH 7 at 4 °C.

Protein concentrations were estimated by using the following values for $A^{1\%}_{280\ nm}$ and molecular weight, respectively: myosin, 5.6 cm^{-1} and 470 000; HMM, 6.5 cm^{-1} and 350 000; actin, 11 cm^{-1} and 42 000; regulated actin, 8.75 cm^{-1} and 63 000; LC2, 6.0 cm^{-1} and 19 000.

The binding of HMM to regulated actin was examined by using an ultracentrifuge to separate free HMM from that bound to actin (Wagner & Weeds, 1979; Wagner & Giniger, 1981). The conditions used were 10 mM imidazole, 4.5 mM $MgCl_2$, 2 mM ATP, 10^{-4} M DTT, and 0.5 mM EGTA, pH 7 at 20 °C. HMM concentration was 1.5 μM, and regulated actin concentration varied from 10 to 160 μM. The 1-mL samples were centrifuged at 160000g for 20 min, and the supernatants were removed. Aliquots were electrophoresed on polyacrylamide gels in the presence of $NaDodSO_4$ (Laemmli, 1970) and in 8 M urea (Perrie et al., 1973). The inorganic phosphate concentration was measured (Rockstein & Herron, 1951) to determine the extent of ATP hydrolysis during the centrifugation. The gels run in $NaDodSO_4$ were stained with Coomassie Brilliant Blue. The intensities of the HMM heavy chains were determined by using a scanning gel densitometer and compared to control HMM samples centrifuged in the absence of actin. The gels run in urea were stained by using the Bio-Rad silver stain kit, and the ratio of digested LC2 (molecular weight 17 000) to LC3 (the 16 500-dalton, alkali 2 light chain) was determined by using a scanning gel densitometer.

The regulated actin-activated ATPases of HMM in the absence of Ca^{2+} were determined under the same conditions as used for the binding experiments. The same conditions were used to determine the regulated actin-activated ATPases in Ca^{2+} except 0.1 mM $CaCl_2$ was used instead of 0.5 mM EGTA and the HMM concentration was only 0.2 μM. The assays were performed as described by Wagner & Weeds (1979) by measuring the amount of inorganic phosphate produced.

Conditions used for examining the interaction of myosin with F-actin and with regulated actin were 20 mM imidazole, 9 mM $MgCl_2$, 5 mM ATP, 100 mM KCl, 10^{-4} M DTT, and either 0.1 mM $CaCl_2$ or 0.5 mM EGTA, pH 7 at room temperature. F-Actin and regulated actin concentrations were 5 μM, and myosin concentration was varied from 0 to 20 μM. Prior to being mixed, myosin was in 0.15 M KCl and 10 mM imidazole, pH 7. Myosin was mixed with ATP and allowed to sit at room temperature for about 5 min. Immediately after the addition of 50 μL of 50 μM F-actin or regulated actin, the samples were centrifuged in an Eppendorf centrifuge at 15000g for 15 min. Aliquots were removed from the supernatants and analyzed by electrophoresis on 5–15% polyacrylamide gels in $NaDodSO_4$. The gels were stained with Coomassie Brilliant Blue, and the amount of actin remaining in the supernatant was compared to uncentrifuged controls by using a scanning gel densitometer. Optical density mea-

[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; HMM, heavy meromyosin; S1, myosin subfragment 1; LC1, light chain 1 (M_r 21 000); LC2, light chain 2 (M_r 19 000); LC3, light chain 3 (M_r 16 500); ATPase, adenosinetriphosphatase.

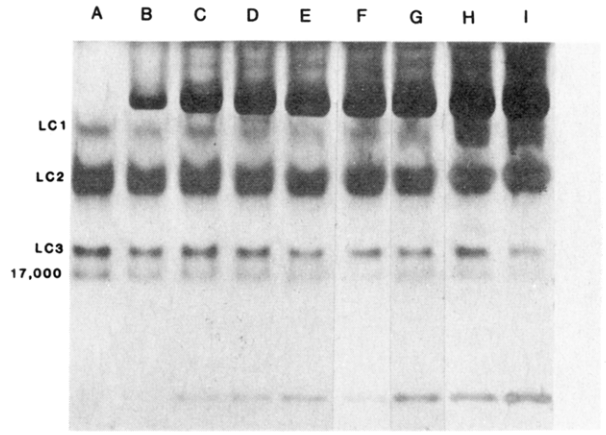

FIGURE 1: Polyacrylamide gel electrophoresis in 8 M urea. Supernatants after centrifugation of 1.5 μM HMM in the presence of various concentrations of regulated actin for 20 min at 160000g were electrophoresed in 8 M urea. Conditions used for the binding were 10 mM imidazole, 4.5 mM $MgCl_2$, 2 mM ATP, 10^{-4} M DTT, and 0.5 mM EGTA, pH 7 at 20 °C. (A) HMM centrifuged in the absence of regulated actin; (B–I) HMM centrifuged in the presence of 7, 14, 20, 26, 35, 50, 70, and 100 μM regulated actin, respectively.

surements were found to be linear over the actin concentrations used.

The K^+-EDTA and Ca^{2+}-ATPases of myosin were determined by using a pH stat as described previously (Wagner & Weeds, 1977). The regulated actin-activated ATPases of myosin were measured in 60 mM KCl, 20 mM imidazole, 5 mM ATP, 9 mM $MgCl_2$, and either 0.1 mM $CaCl_2$ or 0.5 mM EGTA, pH 7 at 25 °C.

Results

HMM Binding to Regulated Actin. HMM containing 15% digested LC2 was mixed with various concentrations of regulated actin in the presence of MgATP and EGTA and centrifuged at 160000g for 20 min to separate free HMM from that bound to regulated actin. The supernatants were examined by electrophoresis in 8 M urea (Figure 1). In urea, the 17 000-dalton fragment of LC2 migrates ahead of LC3 (the 16 500-dalton, alkali-2 light chain), but in $NaDodSO_4$, they comigrate. The gels were silver stained as the samples contained less than 0.2 μg of the 17 000-dalton fragment. The only two subunits of HMM which are well resolved in urea from those of regulated actin (a small fraction of which does not sediment) are LC3 and the 17 000-dalton fragment of LC2. As the regulated actin concentration was increased, the amount of digested LC2 remaining in the supernatant decreased more rapidly than did LC3. The ratio of LC3 to digested LC2 increased from 1.75:1 for HMM centrifuged in the absence of actin to 3.4:1 and 4.2:1 for HMM which remained in the supernatant after centrifugation in 100 and 160 μM regulated actin, respectively. The decrease in the amount of LC3 in the supernatant results from both HMM containing digested LC2 and HMM containing intact LC2 binding to regulated actin. The more rapid decrease in the amount of digested LC2 relative to that of LC3 shows that in MgATP and EGTA, HMM containing digested LC2 binds more tightly to regulated actin than does HMM containing intact LC2.

The ratio of digested LC2 to LC3 was used to calculate the ratio of intact to digested LC2 for the HMM which did not bind to regulated actin. This ratio increased from 5.7:1 for HMM centrifuged in the absence of actin to 14.3:1 for HMM centrifuged in 160 μM regulated actin. Aliquots of these supernatants were also electrophoresed in $NaDodSO_4$. The intensities of the heavy chain bands were measured, and the

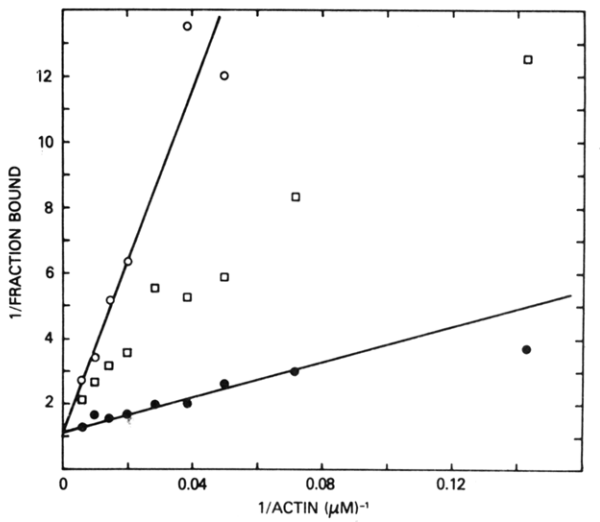

FIGURE 2: Binding of HMM to regulated actin in the presence of MgATP and EGTA. Conditions were as given in Figure 1. Supernatants after centrifugation of 1.5 μM HMM in the presence of various concentrations of regulated actin were electrophoresed on polyacrylamide gels in the presence of $NaDodSO_4$ and in the presence of 8 M urea. The intensities of the HMM heavy chains on the gel run in $NaDodSO_4$ were determined by using a scanning gel densitometer, and they were used to calculate the fraction of HMM bound to regulated actin (□). The ratios of digested LC2 to LC3 were determined from their intensities on the gel run in urea (Figure 1). These ratios were used to calculate the ratios of intact to digested LC2 for the HMM remaining in the supernatants. The ratio of intact to digested LC2 and the concentration of HMM remaining in the supernatant were used to calculate the concentrations of HMM containing digested LC2 and the concentrations of HMM containing only intact LC2 which remained in the supernatants. These values were used to determine the fraction of HMM containing only intact LC2 (○) and the fraction of HMM containing digested LC2 (●) which bound to regulated actin.

concentration of HMM which did not bind to regulated actin was calculated. These concentrations and the ratios of intact to digested LC2 were used to calculate the concentrations of HMM containing only intact LC2 and the concentrations of HMM containing digested LC2 which remained in the supernatant after centrifugation with regulated actin. It was assumed that 17 000-dalton fragments of LC2 were randomly distributed and that HMM containing either 1 or 2 mol of digested LC2 had the same binding properties (28% of the original HMM contains either 1 or 2 mol of digested LC2). It was also necessary to assume that HMM containing intact LC2 and HMM containing digested LC2 had the same LC3 composition. The fractions of these two types of HMM bound at the different regulated actin concentrations were calculated and plotted as shown in Figure 2. Both appear to extrapolate to about 100% bound at infinite actin concentration. HMM containing digested LC2 bound to regulated actin in MgATP and EGTA with an association constant, K_a, of approximately 4×10^4 M^{-1}, and HMM containing only intact LC2 had a K_a of approximately 4×10^3 M^{-1}.

Regulated Actin-Activated ATPases. Another measure of the affinity of HMM for actin is K_{app}, the actin concentration required to achieve half the maximum ATPase rate, V_{max}. The regulated actin-activated ATPase of HMM containing 90% intact LC2 was compared to that of HMM containing only 30% intact LC2. The heavy chains and alkali light chains, LC1 and LC3, of these two HMM preparations appear to be the same. In the absence of Ca^{2+}, the regulated actin-activated ATPases of both HMM preparations have a V_{max} of 0.2 s^{-1}, but the values for K_{app} were quite different (Figure 3). HMM with mostly digested LC2 had a K_{app} of 20 μM, while HMM

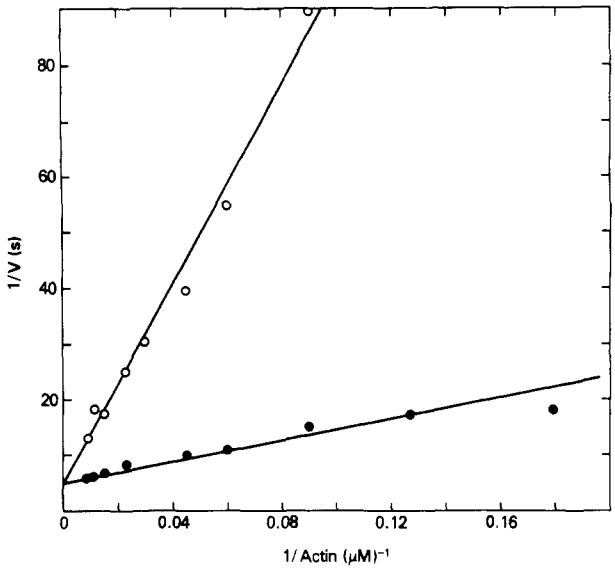

FIGURE 3: Regulated actin-activated ATPase of HMM in EGTA. Conditions were the same as those given in Figure 1. (●) HMM containing 30% intact LC2; (○) HMM containing 90% intact LC2.

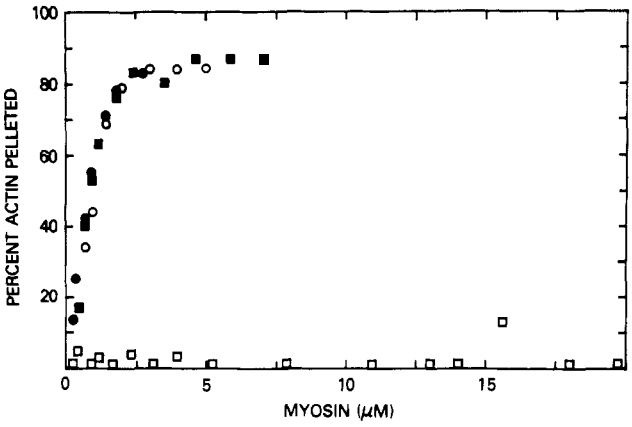

FIGURE 4: Cross-linking F-actin and regulated actin by myosin. Conditions were 20 mM imidazole, 9 mM $MgCl_2$, 5 mM ATP, 100 mM KCl, 10^{-4} M DTT, and either 0.1 mM $CaCl_2$ or 0.5 mM EGTA, pH 7 at room temperature: 5 μM F-actin in EGTA (○) and in Ca^{2+} (●); 5 μM regulated actin in EGTA (□) and in Ca^{2+} (■).

with mostly intact LC2 had a K_{app} of around 190 μM. This difference in K_{app} cannot be attributed to mixing difficulties as the greatest differences in ATPase rates are at low regulated actin concentrations (Figure 3). As V_{max} is the same for the two HMM preparations, the difference in K_{app} values is not due to varying amounts of HMM with unregulated ATPase activity. Also, the K_{app} values are too low to reflect interactions with actin not regulated by troponin–tropomyosin. In Ca^{2+}, the regulated actin-activated ATPases of both HMM preparations had V_{max} values of 6.7 s^{-1}. The K_{app} of HMM containing mostly digested LC2 was 20 μM, while that of HMM containing mostly intact LC2 was 50 μM. Another HMM preparation which contained 85–90% intact LC2 had a K_{app} in Ca^{2+} of 25 μM (Wagner & Stone, 1983a).

Myosin Cross-Linking Regulated Actin. When 5 μM F-actin or regulated actin was centrifuged at 15000*g* for 15 min, less than 5% sedimented. In the presence of increasing amounts of myosin, the amount of actin sedimented increased (Figure 4). Although myosin is filamentous at this ionic strength, μ = 0.14 M, only about 25% of it sedimented after 15 min at 15000*g* in the absence of actin. When myosin filaments bind to actin, large cross-linked aggregates are formed which sediment easily. When F-actin was used, the

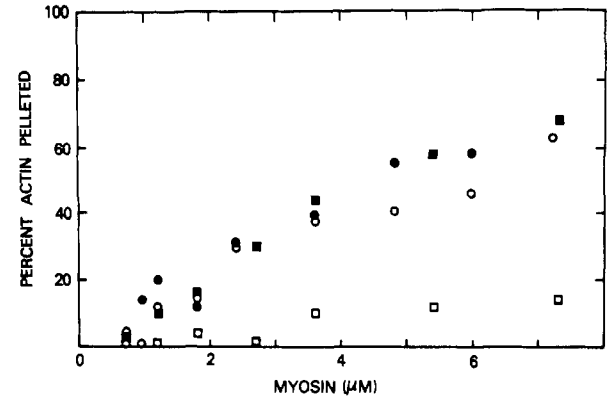

FIGURE 5: Effect of LC2 on the cross-linking of regulated actin by myosin. Conditions were the same as those given in Figure 4 except the KCl concentration was 50 mM. Approximately 30% of the LC2 was removed from myosin by incubation at 37 °C in EDTA and ATP. Cross-linking by this depleted myosin in EGTA (○) and in Ca^{2+} (●). LC2 was added back to this depleted myosin. Cross-linking by this recombined myosin in EGTA (□) and in Ca^{2+} (■).

fraction sedimented by myosin in MgATP was the same in the presence and absence of Ca^{2+} (Figure 4). Approximately half the 5 μM F-actin was sedimented by 0.9 μM myosin. This binding was not due to the formation of rigor bonds, as more than 2 mM ATP remained at the end of the centrifugation, nor did it result from superprecipitation, as it took more than 20 min for superprecipitation to occur, when 5 μM F-actin and 4 μM myosin were used. At the same ionic strength but in the absence of ATP, half the 5 μM F-actin was sedimented by 0.14 μM myosin.

The amount of regulated actin sedimented by myosin in the presence of MgATP was much less in EGTA than in Ca^{2+} (Figure 4). In Ca^{2+}, the fraction of regulated actin pelleted was comparable to the results obtained with F-actin; approximately half the 5 μM regulated actin was sedimented by 0.9 μM myosin. In EGTA, less than 15% of the regulated actin sedimented even in 20 μM myosin.

The effect of LC2 on the Ca^{2+}-sensitive cross-linking of regulated actin was also examined. Myosin was 30% depleted of LC2 by incubation at 37 °C in EDTA and ATP (Wikman-Coffelt et al., 1979). LC2 removal does not appear to be random, but rather the first light chain is dissociated more easily than the second (Weeds & Lowey, 1971; Holt & Lowey, 1975; Wikman-Coffelt et al., 1979). Thus, 30% depletion of LC2 means that 60% of the myosin contains only one LC2. This LC2-depleted myosin had K^+-EDTA and Ca^{2+}-ATPase activities which were 90% of those of control myosin. However, this treatment substantially altered the interaction of myosin with regulated actin. At the ionic strength used for control myosin, 7.2 μM LC2-depleted myosin pelleted only 25% of the regulated actin in the presence of MgATP and Ca^{2+}. This decrease in cross-linking was caused by the 37 °C incubation and not by LC2 removal per se since when LC2 was added back to the LC2-depleted myosin, the amount of regulated actin cross-linked in the presence of Ca^{2+} and MgATP did not increase.

Because of the apparent decrease in the affinity of myosin for regulated actin caused by the 37 °C incubation, it was necessary to perform the cross-linking experiments with LC2-depleted myosin and with the recombined myosin at an ionic strength of 90 mM instead of the 140 mM used for native myosin. At this lower ionic strength, reasonable amounts of cross-linking were obtained (Figure 5). LC2-depleted myosin cross-linked regulated actin in MgATP almost as well in the absence of Ca^{2+} as in its presence; approximately half the 5

μM regulated actin was sedimented by 4 μM LC2-depleted myosin in Ca^{2+} and by 6 μM LC2-depleted myosin in EGTA. Recombination of the LC2-depleted myosin with LC2 resulted in an increase in the Ca^{2+} sensitivity of this cross-linking (Figure 5). In Ca^{2+}, approximately 4 μM recombined myosin pelleted half the 5 μM regulated actin, about the same as that obtained with LC2-depleted myosin in Ca^{2+}. In EGTA, 7.3 μM recombined myosin sedimented only 15% of the regulated actin, much less than the 63% pelleted by a comparable concentration of LC2-depleted myosin under identical conditions.

The regulated actin-activated ATPase activities of the depleted and recombined myosins in the presence of Ca^{2+} and 10 μM regulated actin were 0.33 s^{-1}, while control myosin which had been dialyzed under the same conditions had an ATPase activity of 0.36 s^{-1}. In EGTA, there was no activation of the ATPase activities of these myosins by regulated actin.

Discussion

HMM prepared by even a mild chymotryptic digestion contains 10–15% digested LC2. This is the type of HMM used by both Wagner & Stone (1983a) and Chalovich & Eisenberg (1984). As shown in Figure 2, the double-reciprocal plot for the binding of this HMM to regulated actin in MgATP and EGTA is not linear. Urea gel electrophoresis was used to distinguish the binding of HMM which contains the 17 000-dalton fragment of LC2 from that of HMM which contains only intact LC2. The double-reciprocal plots for the binding of these two types of HMM are reasonably linear (Figure 2). HMM containing only intact LC2 bound 10-fold more weakly than HMM containing digested LC2. No effort was made to distinguish between HMM containing 1 or 2 mol of digested LC2. The association constant for HMM containing digested LC2 obtained from the data shown in Figure 2 is 4×10^4 M^{-1}. This is similar to the 2.6×10^4 M^{-1} found for the binding of HMM containing 60% digested LC2 to regulated actin in EGTA, when no attempt was made to distinguish between HMM containing intact and digested LC2 (Wagner & Stone, 1983a). At the ionic strength used in the experiments described here, the affinity of HMM for regulated actin in Ca^{2+} is $(2–4) \times 10^4$ M^{-1} whether or not LC2 is digested (Wagner & Stone, 1983; Chalovich & Eisenberg, 1984). Thus, as concluded by Wagner & Stone (1983a), the binding of HMM containing digested LC2 to regulated actin in MgATP appears to be Ca^{2+} insensitive, while HMM containing intact LC2 binds more weakly in EGTA than in Ca^{2+}.

Depending on conditions, the K_{app} of the actin-activated ATPase of S1 or HMM is either similar to or less than the dissociation constant for the binding of S1 or HMM to actin in the presence of MgATP (Eisenberg et al., 1972; Wagner & Weeds, 1979; Stein et al., 1981; Wagner & Giniger, 1981; Chalovich & Eisenberg, 1982). However, under all conditions, the value for K_{app} depends on the affinity of S1 or HMM for actin. In the absence of Ca^{2+}, the K_{app} of the regulated actin-activated ATPase of HMM containing 90% intact LC2 is 9-fold weaker than that of HMM containing only 30% intact LC2. This also indicates that cleavage of LC2 to a 17 000-dalton fragment substantially increases the affinity of HMM for regulated actin in the presence of MgATP and EGTA. While these two HMM preparations have similar affinities for regulated actin in Ca^{2+} and MgATP (Wagner & Stone, 1983a), the K_{app} of the regulated actin-activated ATPase in Ca^{2+} of one HMM preparation containing mostly intact LC2 was 2–3-fold weaker than that of HMM containing mostly digested LC2. While this result suggests that LC2 cleavage may cause a small increase in the affinity of HMM for regulated actin in Ca^{2+}, another HMM preparation which contained mostly intact LC2 had a K_{app} about equal of that of HMM containing mostly digested LC2.

Both the binding experiments and the regulated actin-activated ATPases show that HMM containing digested LC2 binds to regulated actin Ca^{2+} insensitively, but how Ca^{2+} sensitive is the binding of HMM which contains intact LC2? The K_a for the binding of this HMM to regulated actin in MgATP varies from 2×10^4 to 4×10^4 M^{-1} in Ca^{2+}, and it is about 4×10^3 M^{-1} in EGTA. The regulated actin-activated ATPase of HMM containing 85–90% intact LC2 has a $1/K_{app}$ value of $(2–4) \times 10^4$ M^{-1} in Ca^{2+} and approximately 5×10^3 M^{-1} in EGTA. Thus, under these conditions, 20 °C and μ = 0.02 M, removal of Ca^{2+} causes a 5–10-fold decrease in the affinity of HMM for regulated actin, somewhat less than the 10–20-fold estimated previously (Wagner & Stone, 1983a). One reason for this lower estimate is the possibility that LC2 cleavage in addition to causing a 10-fold increase in the affinity of HMM for regulated actin in the absence of Ca^{2+} may cause about a 2-fold increase in the presence of Ca^{2+}. Chalovich & Eisenberg (1984) found only a 3-fold difference in the affinity of HMM for regulated actin in the presence of absence of Ca^{2+}. However, if they assume that the fraction of HMM which contains digested LC2 binds Ca^{2+} insensitively, they find that the HMM which contains intact LC2 binds 5-fold more weakly in EGTA than in Ca^{2+} (J. Chalovich and E. Eisenberg, personal communication).

The cross-linking of F-actin by filamin, fodrin (brain spectrin), macrophage actin binding protein, actinogelin, and myosin in the absence of ATP has been examined by using low-speed centrifugation (Brotschi et al., 1978; Mimura & Asano, 1979; Glenney et al., 1982). This approach has been extended to examine the binding of myosin filaments to actin in the presence of MgATP. The ionic strength used for these cross-linking experiments, μ = 0.14 M, is closer to physiological than that used for the HMM binding experiments, μ = 0.02 M.

The ratio of myosin added to actin sedimented in the absence of ATP, 1 myosin head to 9 actin monomers, indicates that many of the myosin heads from one myosin filament bind to the same actin filament and, therefore, do not contribute to cross-linking actin filaments. If the myosin heads distribute differently in the presence and absence of MgATP, the extent of actin cross-linking in MgATP cannot be used to calculate an association constant. Whether or not an association constant can be determined from the cross-linking experiments, there is clearly a difference in the binding of myosin in MgATP to regulated actin in the presence and absence of Ca^{2+}. This does not result from Ca^{2+} binding to myosin, as the sedimentation of F-actin by myosin is Ca^{2+} insensitive. The simplest interpretation is that the affinity of myosin for regulated actin is lower in the absence of Ca^{2+} than in its presence.

Myosin partially depleted of LC2 by incubation at 37 °C in EDTA and ATP did not cross-link regulated actin as well as did control myosin. As readdition of LC2 did not reverse this, the reduction in binding affinity results from the incubations at 37 °C and not LC2 removal. Because of this reduced affinity, the cross-linking experiments with depleted and recombined myosins were performed at 0.09 M ionic strength. The cross-linking observed at this lower ionic strength does not appear to result from the formation of rigor bonds by myosin denatured during the 37 °C incubation, as rigor bonds would have also resulted in cross-linking at the higher ionic strength. This LC2-depleted myosin cross-linked regulated actin in MgATP almost as well in EGTA as in Ca^{2+}; it required about 50% more LC2-depleted myosin to precipitate

the regulated actin in EGTA than it did in Ca^{2+}. This result indicates removal of one LC2 from myosin (60% of this myosin contains one LC2) is sufficient to cause loss of this Ca^{2+} sensitivity. Recombination of this depleted myosin with LC2 partially restored the Ca^{2+} sensitivity of the cross-linking of regulated actin. Thus, as observed with HMM (Wagner & Stone, 1983a), LC2 is required for the Ca^{2+}-sensitive binding of myosin to regulated actin in the presence of MgATP. As the regulated actin-activated ATPase of LC2-depleted myosin is Ca^{2+} sensitive, a subsequent step in the ATPase cycle is inhibited in the absence of Ca^{2+}.

The following scheme for the actomyosin ATPase contains only those steps which are important for the discussion as to where regulation by troponin–tropomyosin occurs. A is actin and M is myosin.

$$\begin{array}{ccccccccc} & & \mathrm{M{\cdot}ATP} & \rightleftharpoons & \mathrm{M{\cdot}ADP{\cdot}P_i} & & & & \\ & & \kappa_1 \downarrow\uparrow & & \kappa_2 \downarrow\uparrow & & & & \\ \mathrm{A{\cdot}M} & \rightleftharpoons & \mathrm{A{\cdot}M{\cdot}ATP} & \rightleftharpoons & \mathrm{A{\cdot}M{\cdot}ADP{\cdot}P_i} & \overset{k_3}{\rightleftharpoons} & \mathrm{A{\cdot}M{\cdot}ADP} & \rightleftharpoons & \mathrm{A{\cdot}M} \end{array}$$

The simplest explanation for the Ca^{2+}-sensitive cross-linking of regulated actin by myosin is that in the absence of Ca^{2+}, troponin–tropomyosin inhibits the binding of myosin to regulated actin; i.e., association constants K_1 and K_2 are greater in the presence of Ca^{2+} than in its absence. The Ca^{2+}-sensitive binding of HMM to regulated actin is consistent with this interpretation. However, it is possible for K_1 and K_2 to be independent of Ca^{2+} and still observe Ca^{2+}-sensitive cross-linking, if in Ca^{2+}, ADP release from the actin–myosin–ADP complex is inhibited. As ADP release from acto-S1 (White, 1977; Marston & Taylor, 1980) is very fast, this inhibition would have to result from some interaction of the cross-bridge with the myosin filament. In the absence of Ca^{2+}, troponin–tropomyosin inhibits P_i release, k_3, and thereby prevents the formation of A·M·ADP (Chalovich et al., 1981; Wagner & Giniger, 1981; Chalovich & Eisenberg, 1982, 1984; Wagner & Stone, 1983a; Inoue & Tonomura, 1982). It could be the formation of this complex which allows for cross-linking of regulated actin at lower myosin concentrations in Ca^{2+} than in EGTA. While this is a possible explanation, the results with LC2-depleted myosin argue against it. Cross-linking by LC2-depleted myosin in the absence of Ca^{2+} is probably due to the formation of actin–myosin–ADP–P_i complexes (Chalovich & Eisenberg, 1982). As LC2-depleted myosin cross-links almost as well in EGTA as in Ca^{2+}, there should be about the same number of myosins bound to regulated actin under both sets of conditions. This similarity suggests that the actin–myosin–ADP complex contributes little to the fraction of myosin bound to regulated actin in Ca^{2+}.

The results presented here and in previous publications (Chalovich et al., 1981; Wagner & Giniger, 1981; Chalovich & Eisenberg, 1982; Inoue & Tonomura, 1982; Wagner & Stone, 1983a) demonstrate that troponin–tropomyosin in the absence of Ca^{2+} inhibits both the attachment of myosin to actin and some subsequent step in the ATPase cycle. Regulation of the actin-activated ATPases of smooth muscle HMM by phosphorylation (Sellers et al., 1982) and of scallop and squid HMM by Ca^{2+} binding to the HMM (Chalovich et al., 1983) also appears to occur both at their binding to actin and at some subsequent step in the ATPase cycle, but at least at low ionic strength, inhibition of binding cannot be the principal mechanism for regulating the actin-activated ATPases of these HMM preparations.

The relative importance of these two sites of inhibition for the regulation of muscle contraction is as yet unknown. Stiffness measurements of relaxed glycerinated skeletal muscle fibers using very rapid stretches at low ionic strength and temperature showed that myosin cross-bridges can attach to the thin filament without developing force (Brenner et al., 1982). However, differences in the X-ray scattering 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 myosin in relaxed glycerinated fibers indicate that most of the myosin cross-bridges are not bound to the thin filament under relaxing conditions. Recent time-resolved X-ray diffraction measurements of intact muscle (Huxley et al., 1984) indicate that upon stimulation tropomyosin movement precedes an increase in the number of cross-bridges attached to actin. This indicates that under relaxing conditions troponin–tropomyosin at least partially inhibits the attachment of myosin to actin.

Acknowledgments

I thank Dr. Claude Klee for her critical reading of the manuscript, Drs. Evan Eisenberg and Joseph Chalovich for helpful discussions, and Janet George for her excellent technical assistance.

Registry No. ATPase, 9000-83-3; MgATP, 1476-84-2; Ca, 7440-70-2.

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Polyclonal Antibody That Recognizes Calcium-Dependent Determinants in *Tetrahymena* Calmodulin†

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ABSTRACT: We report here that a precipitating antibody prepared against *Tetrahymena pyriformis* calmodulin recognizes calcium-dependent determinants in the native protein. The ability of the antibody to precipitate ^{35}S-labeled *Tetrahymena* calmodulin in direct radioimmunoassays was enhanced at least 3-fold in the presence of calcium. Competitive radioimmunoassay using homogeneous preparations of endogenously ^{35}S-labeled *Tetrahymena* calmodulin and protein A–Sepharose-purified immunoglobulin G demonstrated that this antibody preparation is specific for protozoan calmodulin. Homogeneous vertebrate, invertebrate, and plant calmodulins, as well as rabbit skeletal muscle troponin C, did not show significant competition with the ^{35}S-labeled *Tetrahymena* protein at concentrations 100-fold greater than that at which the homologous unlabeled *Tetrahymena* calmodulin produced 50% competition. A cyanogen bromide digest of *Tetrahymena* calmodulin also showed partial competition with the intact ^{35}S-labeled protein, but only in the presence of calcium. The *major* antigenic determinants were localized to the carboxyl-terminal half of the molecule by immunoassay of limited trypsin fragments of *Tetrahymena* calmodulin. The antibody bound native calmodulin complexed to bovine brain phosphodiesterase (EC 3.1.4.17) but failed to recognize the *Tetrahymena* calmodulin carboxyl-terminal fragment (76–147) when complexed to the enzyme.

Calmodulin is a widely distributed, highly conserved protein that mediates the calcium-dependent regulation of many enzymes [for a review, see Klee & Vanaman (1982)]. Despite this high degree of sequence homology, several laboratories have succeeded in producing antibodies against mammalian calmodulin (Andersen et al., 1978; Dedman et al., 1978; Wallace & Cheung, 1979; Van Eldik & Watterson, 1981; Hansen & Beavo, 1983; Pardue et al., 1983). Mammalian calmodulin has been successfully employed as an antigen as the native protein (Andersen et al., 1978; Dedman et al., 1978), in a chemically modified form (Wallace & Cheung, 1979; Van Eldik & Watterson, 1981), or in enzyme complexes (Hansen & Beavo, 1983). Recently, in vitro immunization also has been used to produce monoclonal antibody of usable titer (Pardue et al., 1983). These antibodies show little specificity for calmodulin from any particular source, and only in limited instances [e.g., see Andersen et al. (1978) and Chafouleas et al. (1979)] do they appear to form precipitating antigen–antibody complexes. By contrast, antibody against native calmodulin from the ciliated protozoan *Tetrahymena pyriformis* is both precipitating and highly specific for protozoan calmodulin (Suzuki et al., 1982; present study). In this paper, we report the characterization of an anti-*Tetrahymena* calmodulin antibody and the ability of this antibody to recognize calcium-dependent determinants on calmodulin and calmodulin–enzyme (bovine brain phosphodiesterase, EC 3.1.4.17) complexes. The localization of the major *Tetrahymena* calmodulin antigenic determinants to the carboxyl-terminal half of the molecule increases the usefulness of the antibody as a tool for studying the interactions of calmodulin with calmodulin binding proteins, both in vitro and in situ. a preliminary report of portions of this work has been presented (McCartney et al., 1982).

Materials and Methods

Materials. Pansorbin (buffered suspension of pickled *Staphylococcus aureus* cells) was purchased from Calbiochem-Behring Corp., La Jolla, CA. Protein A and *N*-succinimidyl 3-(4-hydroxyphenyl)propionate (Bolton–Hunter reagent) were purchased from Sigma Chemical Co., St. Louis, MO. Cyanogen bromide (CNBr)[1] and methyl *p*-hydroxybenzimidate (Wood's reagent) were purchased from Pierce Chemical Co., Rockford, IL. Noble agar was purchased from Difco Laboratories, Detroit, MI. $Na^{125}I$ and $H_2{}^{35}SO_4$ were

† From the Department of Microbiology and Immunology (J.E.M. and T.C.V.) and the Department of Physiology (J.J.B.), Duke University Medical Center, Durham, North Carolina 27710. *Received February 10, 1984.* This research was supported by National Institutes of Health Grants NS 10123 (T.C.V.) and 2T32 AI-07148 (J.E.M., training grant) and by National Science Foundation Grant PCM-8112259 (J.J.B.).

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[1] Abbreviations: CNBr, cyanogen bromide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; RIPA, radioimmunoprecipitation assay; PDE, bovine brain 3′,5′-cyclic nucleotide phosphodiesterase (EC 3.1.4.17); CaM, calmodulin; IgG, immunoglobulin G; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography; BSA, bovine serum albumin; DPT, diazophenyl thio ester; APT, aminophenyl thio ester; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

0006-2960/84/0423-5956$01.50/0