INDUCTION OF CONTRACTILE ACTIVITY BY RIGOR COMPLEXES IN MYOFIBRILS

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SUMMARY

The relation between ATPase rate and substrate concentration was investigated for myofibrils with varying amounts of added HMM. There was a biphasic, 3 to 5-fold increase in ATPase in the absence of Ca^{++}. In the absence of added HMM, the peak activity occurred at $\leq$ 0.1 mM MgATP. With increasing concentrations of HMM, the position and magnitude of the ATPase peak shifted to larger substrate concentrations and higher rates. The cofactor activity of regulated actin in myofibrils is activated to a similar degree by Ca^{++} as by HMM (rigor links). SDS gel electrophoretic patterns of myofibrils mixed with HMM indicated the soluble HMM binds to myofibrils at 0.1 mM MgATP and is dissociated at higher MgATP concentrations. Thus, in well-regulated myofibrils in the absence of Ca^{++} actin cofactor activity can be activated by rigor complexes.

In the absence of Ca^{++} and below 0.1 mM MgATP, ATPase is partially activated in myofibrils (1), acto-HMM (2) and acto-S1 (3) and tension is evoked in single fibers (4,5). As the substrate concentration is increased above 0.1 mM MgATP, these indicators of contractile activity are increasingly inhibited. The transitory increase in ATPase activity observed in the regulated acto-S1 system has been attributed to the activation of a functional unit on the thin filament by a threshold number of rigor complexes (3,6). The basis for this hypothesis is the observation that the turning "on" and "off" of actin-activated ATP hydrolysis is not a unique function of substrate concentration. Instead the MgATP concentration at which the transitory peak occurred increased with successively larger S1/actin ratios, as required by a threshold ratio of rigor complexes to actin needed for activation. In the absence of Ca^{++}, this cooperative potentiation was observed within the acto-S1 system at an S1/actin ratio of 10/1, whereas at an S1/actin ratio of 1/9 no potentiation was observed (3). The overall ratio of S1/actin in vertebrate myofibrils, single fibers and intact muscle is about 1/2. At this S1/actin ratio potentiation of actin-cofactor activity by rigor complexes has not been demonstrated in the completely soluble acto-S1 system even in the presence of Ca^{++}. With an overall ratio of S1 to actin of about 1/2, the interaction of between 6 and 12 S1 molecules with 14 actin monomers is compatible with structural constraints in myofibrils (7). It is possible that the very specific arrangement

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of actin and myosin in the myofilament increases their probability of interaction and thus increases their "effective" concentrations. We have performed experiments that address the question of whether the natural myofibrillar molar ratios of myosin to actin together with structural constraints are compatible with cooperative potentiation of actin cofactor activity by rigor complexes.

MATERIALS AND METHODS

Myofibrils were prepared following the method of Etlinger et al. (8). We find the procedure employed in preparing the myofibrils affects the ATPase characteristics and Ca^{++} sensitivity. For the experiments described below, muscles from the legs of Rana pipiens were quickly dissected and minced with scissors in cold pyrophosphate relaxing buffer (100 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM dithiothreitol (DTT), 2 mM $Na_4P_2O_7$, 10 mM imidazole, pH 7.0). The mince was homogenized in a Dounce homogenizer in the same solution. The homogenate was filtered through two layers of cheese cloth, centrifuged at 800 g for 5 minutes and the pellet was washed several times in a similar buffer (same composition as pyrophosphate relaxing buffer except pyrophosphate was omitted). This procedure was repeated daily because aged myofibrils had decreased Ca^{++} sensitivity.

While it might have been desirable to prepare myosin and HMM from frogs for the combination experiments, the properties of this preparation and procedures used for the extraction are not well documented. We chose instead to prepare myosin from rabbit. We felt this would not be a problem because frog and rabbit myofibril ATPase activities are similar. Myosin was prepared by the method of Kielly and Harrington (9) using back and hind-limb muscles of rabbits. HMM was prepared by the method of Lowey and Cohen (10). HMM was further purified by precipitation in 2.5 M ammonium sulfate and dialyzed against 0.2 M KCl, 0.01 M imidazole, 1 mM DTT, pH 7.1, 4°C.

ATPase activity was detected by creatine production (11) in the presence of creatine phosphokinase and creatine phosphate, or by inorganic phosphate production (12). Myofibrils were added to a total of 3 ml buffer (0.1-0.2 mg/ml), which in the absence of Ca^{++} contained: 80 mM KCl, 10 mM imidazole, 1 mM $MgCl_2$, 1 mM EGTA, ATP between 3 μM and 3 mM, 2 mg/ml creatine phosphokinase and 8 mM phosphorylcreatine. In some experiments Ca^{++} concentration was varied by adding Ca-EGTA buffers (13). Standard incubations, begun by adding enzymes, were 1.5 min at 20°.

The substrate dependence of myofibril ATPase was observed at four different ratios of HMM to myofibrils and are expressed as molar ratios of added HMM to actin contained in the myofibrils. The molar ratios are calculated assuming the mass percent of actin in myofibrils is about 20 (7) and the molecular weights of actin and HMM are about 47,000 and 350,000 daltons respectively. Myofibrils were preincubated with HMM on ice for 30 minutes, and added to the incubation mixture at 20°.

SDS polyacrylamide gel electrophoresis was carried out according to the method of Weber and Osborn (14) with 6% acrylamide. The effect of ATP on binding of HMM to myofibrils was studied by incubating myofibrils (1.3 mg/ml) and HMM (2 mg/ml) in buffer (90 mM KCl, 1.5 mM EGTA, 0.5 mM DTT, 10 mM imidazole, 1.5 mM $MgCl_2$, varying ATP) for 30 minutes. Myofibrils were then centrifuged at 2,000 g for 5 minutes, supernatant poured off and myofibrils washed with 5 ml of the same buffer. The myofibrils were pelleted and then dissolved by heating in 1 ml 1% SDS, 1% β-mercaptoethanol, 10 mM phosphate buffer before electrophoresis. The gels were stained with Coomassie Blue. The presence of HMM bands not otherwise present in the myofibrillar electrophoretic pattern indicated HMM binding.

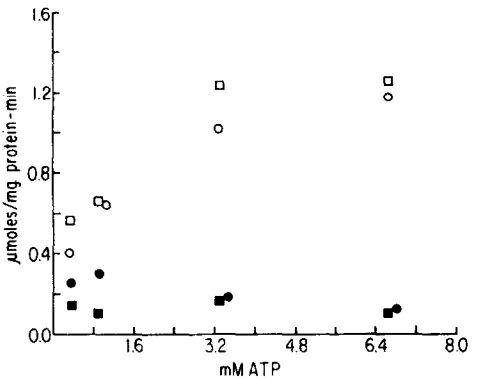

Figure 1: ATPase activity in the presence (○, □ 0.1 mM $CaCl_2$) and absence of Ca^{++} (●, ■ 1 mM EGTA) is shown for two different myofibril preparations, (○,●; □,■). Other assay conditions, 80 mM KCl, 10 mM imidazole, 1 mM $MgCl_2$, pH 7, 20°C, myofibril concentration 0.1-0.2 mg/ml.

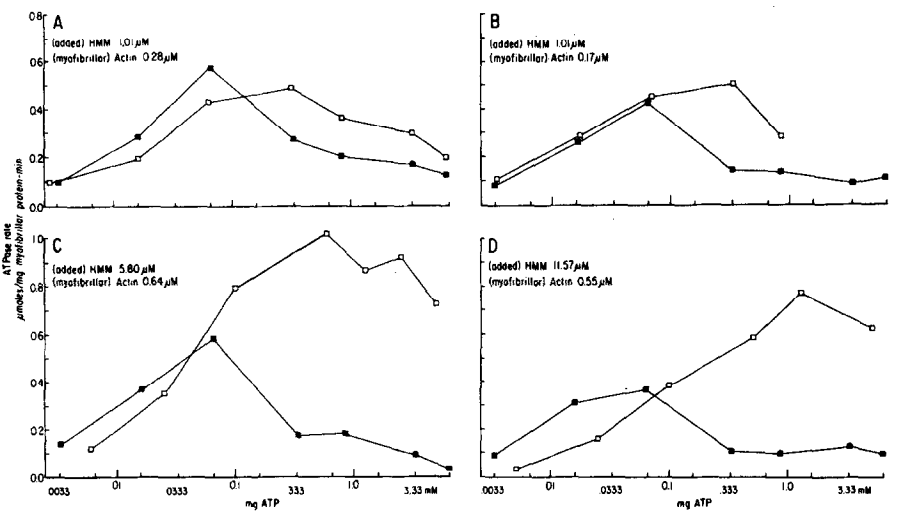

Figure 2: Each figure (a-d) shows the ATPase activity of myofibrils at a different molar ratio of (added) HMM/(myofibrillar) actin, □ . The filled squares, ■ , indicate the ATPase activity of the same myofibril preparation, in the absence of added HMM, obtained on the same day as the combination experiment. Assay conditions: 80 mM KCl, 10 mM imidazole, 1 mM $MgCl_2$, 1 mM EGTA, 8 mM creatine phosphate, 2 mg/ml creatine phosphokinase, pH 7, 20°C. The final concentrations of myofibrils and HMM in the assay are: A - HMM/actin = 4, 0.065 mg/ml myofibrils; B - HMM/actin = 6, 0.04 mg/ml myofibrils; C - HMM/actin = 9, myofibrils 0.15 mg/ml myofibrils; D - HMM/actin = 21, 0.13 mg/ml myofibrils.

RESULTS

The ATPase activity of the myofibrils as a function of substrate concentration in the presence and absence of Ca^{++} is shown in Fig. 1. The maximal ATPase in the presence of Ca^{++} is about 10x greater than in its absence.

Figs. 2 a-d show the result of the combination experiments at four different ratios of added HMM to myofibrillar actin. Each combination experiment is compared with ATPase activity study made with the same myofibrils from which HMM was omitted. The peak activity in the absence of added HMM occurred at about 60 μM MgATP and was between 3 and 5 times greater than the ATPase rate at higher and lower ATP concentrations. The activity of HMM alone has been subtracted from the ATPase activity of myofibrils in the presence of the two highest concentrations of HMM (Figs. 2c, 2d). Addition of HMM shifted the peak myofibrillar ATPase activity to higher ATP concentrations, and the maximal rate increased at the three higher molar ratios HMM/actin. At an HMM/actin ratio of 20/1 the peak myofibril ATPase activity increased 10 times over that of myofibrils alone, an increase as large as obtained by Ca^{++} activation. The results strongly suggest the added HMM affects the Mg^{++} activated ATPase characteristics of myofibrils in a predictable manner, increasing both the magnitude of the myofibril ATPase activity and the ATP concentration at which the peak activity occurs.

The actual HMM concentration in proximity to the actin filaments in the myofibrils is not known since myofibrils are insoluble organelles. While myofibrils are finely dispersed in a test tube, there is still a possible diffusion barrier within the myofibril. In order to conclude that rigor complexes, formed between soluble HMM and myofibrillar actin, affect the myofibrillar ATPase as described above, it is necessary to show that added HMM indeed binds to the myofibrils. In Fig. 3 from left to right appear patterns of myofibrils, HMM, and myofibrils + HMM combined and washed, in the absence of Mg^{++} and presence of ATP, respectively. The HMM pattern is clearly present in the myofibrils combined with HMM in the absence of ATP and the pattern is absent in the gels of myofibrils combined with HMM in the presence of 5 mM MgATP. HMM bands appear to exhibit preferential binding. In particular, the 61,000 dalton component is greatly reduced in intensity with respect to the 78,000 and 81,000 dalton components. In Fig. 3 are also shown the gel patterns of myofibrils incubated with HMM in the presence of increasing concentrations of ATP. Between 0 and 5 μM ATP, HMM appears maximally bound to the myofibrils; with 100 μM ATP the intensity of the HMM bands has decreased, indicating decreased binding to the myofibrils with increased ATP concentration.

The observation that the highest ATPase rate in the presence of added HMM did not exceed the rate with Ca^{++} activation of myofibrils alone suggests that the "potentiated state" (15) is not observed in myofibrillar preparations. Myo-

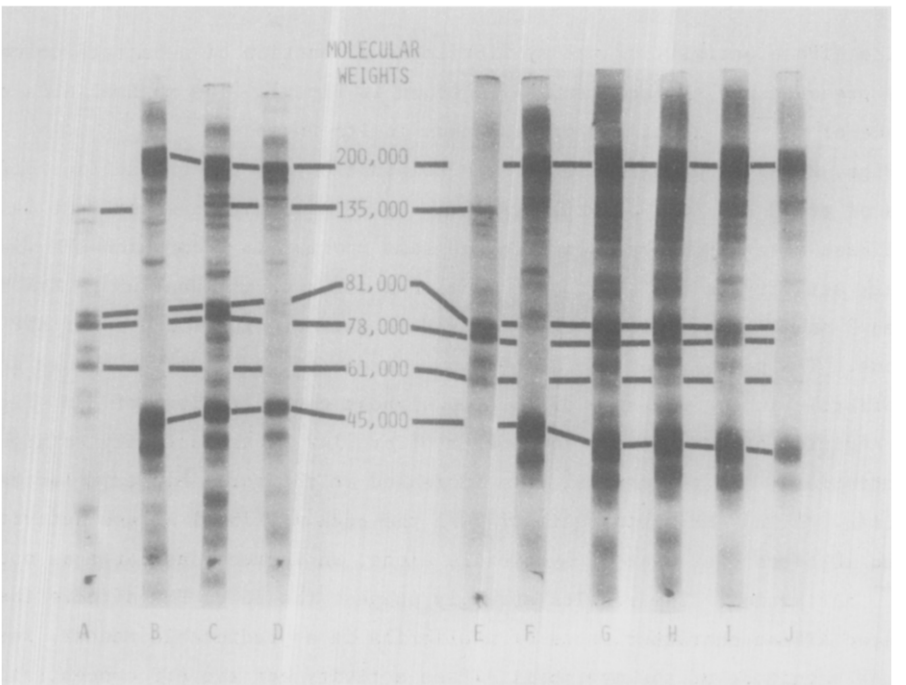

Figure 3: SDS gel (6% polyacrylamide) electrophoretic patterns of HMM, myofibrils and their combination. A - 20 µg of HMM; B - 30 µg myofibrillar protein; C - HMM + myofibrils, and washed in the absence of ATP; D - HMM + myofibrils and washed with 5 mM ATP. Gels C and D contain 30 µg myofibrillar protein and a possible total of 50 µg HMM if all HMM remained bound. E - 20 µg of HMM; F - 50 µg myofibrillar protein (a different preparation from B). G - J - Myofibrils combined with HMM were washed with increasing concentrations of ATP (gel G, no ATP; H, 5 µM; I, 100 µM and J, 1000 µM ATP). The prominent HMM bands that appear in gels F and G are less intense in gels H and I and absent in gel J. Gels F to J contained 80 µg myofibrillar protein and a possible total of 80 µg HMM if all the HMM remained bound.

fibrillar ATPase rates were measured in the presence of Ca^{++} with and without added HMM as a function of substrate concentration (Fig. 4). The molar ratio of added HMM to myofibrillar actin in these experiments was 3/1. At a Ca^{++} concentration of 5.6×10^{-7} M (not shown), which is near the threshold for Ca^{++} activation of myofibrillar ATPase, the added HMM caused the shifts in the transitory ATP peak similar to that observed in the absence of Ca^{++} (similar to Fig. 2). At saturating Ca^{++} concentrations (3×10^{-6} M) there was a monotonic increase in myofibrillar ATPase rate with and without added HMM (Fig. 4 - circles). The shape of these curves is expected on the basis of Ca^{++} activation of thin filaments. Since acto-HMM ATPase is inhibited at high ionic strengths, such as used in our myofibrillar incubations, the experiment was repeated at low ionic strength

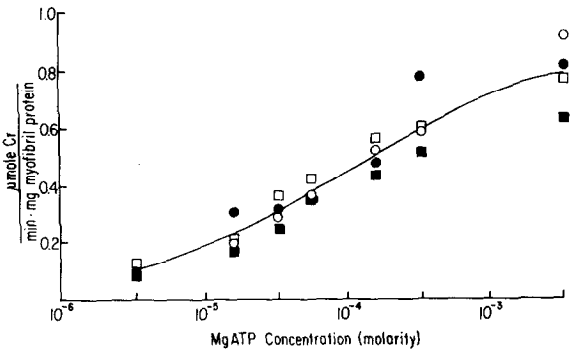

Figure 4: ATPase activity of myofibrils (●,■) and myofibrils + added HMM (○,□) is shown as a function of MgATP at saturating Ca^{++} concentration (3 x 10^{-6} M) at high and low ionic strengths. The molar ratio of added HMM to myofibrillar actin was 3. High ionic strength incubation medium (●,○) contained: 80 mM KCl, 10 mM MOPS, pH 7.0, 3 mM $MgCl_2$, 4 mM EGTA with added Ca^{++} to achieve free Ca^{++} concentration of 3 x 10^{-6} M, 2 mg/ml creatine phosphokinase, 8 mM phosphorylcreatine, and ATP between 3 μM and 3 mM. Low ionic strength incubation medium (■,□) was high ionic strength incubation medium without KCl.

with indistinguishable results (Fig. 4 - squares). SDS gel electrophoretic studies of myofibrils incubated with HMM indicated binding of HMM to myofibrils at both ionic strengths in the presence of Ca^{++}. Thus, under the conditions studied (pCa >8, 6.5 and 5.5; high and low ionic strength) it was not possible to observe an activation of actin cofactor activity in myofibrils by addition of HMM any greater than that observed by Ca^{++} activation.

DISCUSSION

We find the transient increase in myofibrillar ATPase activity in the absence of Ca^{++} is a relatively large effect, beyond the range of error that can be ascribed to assay procedure. Our results support the view that rigor links, in the absence of Ca^{++}, can activate actin cofactor activity in myofibrils at the existing *in situ* ratios of myofibrillar myosin to actin. Our evidence is similar to that used to support the hypothesis of rigor link potentiation in soluble acto-S1: the transient peak in ATPase is not a unique function of ATP concentration but rather is sensitive to the HMM/actin ratio. As in the soluble system, when the ratio of HMM to myofibrillar actin was artificially increased, the position of the ATPase peak shifted to higher ATP concentrations and the height of the peak increased.

SDS polyacrylamide gel electrophoresis showed soluble HMM was capable of binding to myofibrils. Furthermore, substantial HMM binding and thus rigor complexes are observed at 100 μM ATP but not at 1 mM ATP. Thus in the absence of Ca^{++} the peak in myofibril ATPase activity occurs at a substrate concentration

low enough (60 μM ATP, according to the results of the electrophoresis experiments) to permit the presence of rigor links.

The HMM molecule contains a number of internal cleavages, and the fragments are held together by noncovalent forces. Balint et al. (16) report all these components are found in a pellet of F-actin that had been incubated with HMM and centrifuged at 100,000 g. No attempt to wash the F-actin free of HMM was reported. We have found preferential binding of only some HMM bands to the myofibrils in ATP-free buffer. A systematic study of this phenomenon is warranted to obtain more information about which portion of the myosin molecule participates in actin binding.

The presence of three states of actin cofactor activity in soluble acto-HMM systems has been postulated (6): "on", "off" and the "potentiated state". Evidence for a "potentiated state" is derived from the observation that in the completely soluble acto-S1 system, the actin cofactor activity of regulated actin may be increased greatly above that observed upon addition of Ca^{++} alone; the increase is attributed to the presence of rigor complexes. However we find the activation of myofibrillar ATPase by Ca^{++} is about the same magnitude as the maximal activation obtained in the absence of Ca^{++} with high (up to 20/1) HMM/actin ratios. Recently, rigor complexes have been shown to alter the binding constant of tropomyosin to F-actin (17). The author suggested that the stoichiometric relation found between the number of rigor complexes present and the amount of tropomyosin bound to actin indicated conformation changes induced in actin by rigor complexes are not propagated to other monomers. Our results are consistent with a model in which the cofactor activity of some actin monomers in the functional unit are turned "on" by the presence of rigor links on other monomers.

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