All Myosin Heads Form Bonds with Actin in Rigor Rabbit Skeletal Muscle†

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ABSTRACT: We have used an enzymatic technique to measure the number of myosin heads bound to actin in a rigor muscle fiber. In 150 mM KCl and in the absence of divalent cations, the ATPase activity (K-ATPase) of myosin is 0.69 s^{-1}; this activity decreases to 0.21 s^{-1} when the myosin is mixed with excess actin. Similar results were found for myosin subfragments. The amount of heavy meromyosin bound to actin under the above assay conditions was assayed by centrifugation. We found that a maximum of one heavy meromyosin bound per two actins with a binding constant close to that determined from the ATPase measurements. The K-ATPase activity of glycerinated fibers was used to determine the fraction of myosin heads bound to actin. At complete filament overlap (sarcomere length = 2.0–2.2 μm) the K-ATPase activity of the fiber was 0.24 s^{-1} (per myosin head), indicating that most myosin heads were bound to actin. As the sarcomere length was increased the K-ATPase activity increased linearly with the decrease in filament overlap, indicating that unbound myosin heads in the fiber have a K-ATPase activity that is similar to that of myosin in solution. The K-ATPase activity of myosin depends strongly on the KCl concentration while that of actomyosin is independent of KCl concentration. The activity of the fibers at complete filament overlap is also independent of KCl concentration, again indicating that most myosin heads are bound in the fiber. Our results indicte that 94–100% of the myosin heads are bound to actin at complete filament overlap.

Muscle fibers contain two sets of interdigitating filaments: the thick filaments, which are composed mainly of myosin, and the thin filaments, whose core consists of a helical array of actin monomers. Each myosin molecule has two "heads" that bridge the interfilament space and interact with actin to produce force. The distance between repeating actin monomers in the thin filament, ~5 nm, does not match that between repeating myosin heads in the thick filament, ~14.3 nm [see Huxley (1972) for a review]. Thus, some myosin heads may not have an actin with which they can interact, and models of insect flight muscle have suggested that approximately one-third to two-thirds of the myosin heads would not be bound to actin in rigor (Offer & Elliot, 1978; Hazelgrove & Reedy, 1978).

Using an enzymatic technique, we have measured the fraction of myosin heads that bind to actin in a rabbit psoas muscle in rigor. The enzymatic technique relies on an effect discovered by Hasselbach (1957) and investigated in some detail by Rizzino et al. (1970) that in the absence of Mg the K-ATPase activity of free myosin is greater than that of myosin bound to actin. We find that the K-ATPase activity of a fiber in which there is complete overlap between actin and myosin filaments is approximately equal to the activity of myosin bound to actin. Additional evidence that all heads are bound to actin in the fiber comes from measurements of the K-ATPase activity as a function of KCl concentration. The dependence of the fiber K-ATPase activity on KCl resembles that of actomyosin rather than myosin. Calculations based on both the absolute magnitude of the K-ATPase activity and its dependence on KCl agree that very few myosin heads are unable to bind to actin in the region where actin and myosin filaments overlap in the fiber. This result has important implications for theories of muscle structure.

Methods

Proteins. Myosin was purified by the method of Tonomura et al. (1966) as modified by Crooks & Cooke (1977). Actin was prepared by the method of Spudich & Watt (1971). S-1[1] was prepared by digestion of myosin (10 mg/mL) with 0.05 mg/mL α-chymotrypsin for 10 min at 25 °C according to the procedure of Weeds & Lowey (1971). The myosin was sedimented, and the crude S-1 in the supernatant was chromatographed on Sepharose 4B. S-1 prepared by chromatography on Sepharose had a higher K-ATPase activity than that prepared by ion-exchange chromatography. S-1 was also prepared by the method of Cooke (1972), using papain to digest myofibrils. HMM was prepared by digestion of myosin (10–15 mg/mL) with α-chymotrypsin (0.025 mg/mL) for 10 min at 20 °C. Myosin was sedimented, and the HMM in the supernatant was used without further purification. Phosphate was assayed by the method of Martin & Doty (1949). Most ATPase values represent single points taken after 15 min of reaction. The time course of the reaction was found to be linear over this interval. ATPase activities were calculated by assuming molecular weights of 460K, 350K, 135K, and 115K for myosin, HMM, papain S-1, and α-chymotryptic S-1, respectively. Binding studies were carried out by mixing actin and HMM in 150 mM KCl, 4 mM EDTA, 4 mM ATP, and 50 mM Tes, pH 7.5. The samples were immediately centrifuged in a Beckman airfuge at $10^5 g$ for 15 min to produce firm pellets of acto-HMM. The amount of protein in the pellet and supernatant was then assayed. The temperature of the rotor was not regulated and was ~23 ± 2 °C. Protein concentrations were assayed by the method of Bradford (1976).

Fibers. Bundles of rabbit psoas fibers were dissected and tied to wooden sticks and incubated in 50% glycerol, 50% 0.12 M KCl, 5 mM $MgCl_2$, 2 mM EGTA, and 20 mM Tes, pH 7.0, at 0 °C overnight. After a change of solution, they were stored at −20 °C for up to 3 months. Fiber bundles, 5–20 fibers, were dissected on a cold stage and glued to two supports. Their sarcomere length was determined by the diffraction of a laser beam and adjusted in the presence of Mg-ATP if desired. The fibers were first washed for 30 s in 150 mM KCl, 4 mM EDTA, and 20 mM Tes, pH 7.5, and then placed in

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[1] Abbreviations used: S-1, myosin subfragment-1; HMM, heavy meromyosin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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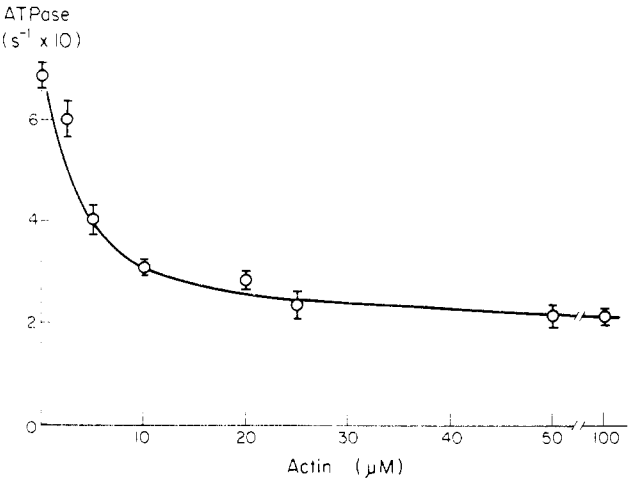

FIGURE 1: K-ATPase rate of myosin (s^{-1}/head) as a function of the actin concentration. The medium contained 150 mM KCl, 4 mM EDTA, 4 mM ATP, and 50 mM Tes, pH 7.5. Assays were carried out at 25 ± 0.5 °C. The assay contained 0.8 μM myosin. Both 1 and 4 mM ATP gave identical results.

4 mL of reaction buffer (150 mM KCl, 4 mM EDTA, 4 mM ATP, and 20 mM Tes, pH 7.5) for 5 min. The fibers were transferred to a second vial containing 4 mL of reaction buffer, and the transfer was repeated up to 5 times. The phosphate in each vial was determined by the method of Martin & Doty (1949), and the values were averaged. The ATPase activity was linear during the time observed. The fiber was then clipped from the supports and sonicated in 0.5 mL of 8 M urea, and the total protein was determined by the method of Bradford (1976). Known amounts of protein, either total fibers or purified myosin, were electrophoresed on 10% polyacrylamide gels in the presence of $NaDodSO_4$ according to the method of Laemmli (1970), the patterns were scanned, and the myosin content of the fibers was determined from the intensities of myosin heavy-chain bands. The heavy-chain band had migrated ~5 mm into the gel, was clearly resolved from other bands, and had an intensity that was linear with the amount of myosin loaded (from 2 to 8 μg). The relative intensities of the myosin heavy-chain bands indicated that 45 ± 3% of the total fiber protein was myosin (by weight).

Results

The ATPase activity of myosin is a strong function of the cation bound to the ATP. In the presence of Mg ions the ATPase activity of myosin is low and is strongly activated by its interaction with actin. When all divalent cations are removed (by addition of EDTA), the ATPase activity of myosin is high in solutions containing K^+ ions; this ATPase activity is inhibited by addition of actin (Hasselbach, 1957; Rizzino et al., 1970), as shown in Figure 1. As increasing amounts of actin are added the ATPase activity decreases, reaching a plateau value that is not zero. Double-reciprocal plots of (activity[myosin] − activity[actomyosin])$^{-1}$ vs. [actin]$^{-1}$ gave approximate straight lines from which apparent binding constants for the binding of actin to myosin in the presence of K-ATP could be calculated as discussed by Rizzino et al. (1970). The solid line in Figure 1 is calculated by using a plateau in ATPase activity of 0.21 s^{-1} and a binding constant of myosin to actin of 5×10^5 M^{-1}, both obtained from the fit to the data in a double-reciprocal plot. Table I shows the ATPase activities of myosin, HMM, and S-1 in the absence of actin and at an actin concentration giving the maximal inhibition. Actin inhibited the ATPase activity of HMM or S-1 in a manner similar to that shown for myosin. The ATPase activity reached the plateau value given in Table I with apparent actin binding constants, determined from double-reciprocal plots, of approximately 10^5 and 7×10^4 M^{-1} for the binding of HMM and S-1, respectively. The ATPase activity of S-1 reached a plateau at a lower value of ATPase activity than that of HMM or myosin. Similar results were found for S-1 prepared with either chymotrypsin or papain, showing that the presence of the 18K dalton light chain is not required for the activities observed here.

Table I: K-ATPase Activity of Myosin, HMM, S-1, and Glycerinated Fibers[a]

	ATPase act. (s^{-1}/head)		
	−actin	+actin	+actin/−actin
myosin	0.69 ± 0.02	0.21 ± 0.01	0.30
HMM	0.59 ± 0.02	0.18 ± 0.01	0.31
S-1	0.68 ± 0.02	0.15 ± 0.01	0.22
fiber[b]	0.85 ± 0.06	0.24 ± 0.02	0.28

[a] The assay conditions were given in the legend to Figure 1. Actin concentration in the +actin column was 50 μM, except for the fiber. In the assays involving actin and myosin, the actin and myosin were first mixed in 0.6 M KCl and then diluted to 150 mM KCl. The addition of 0.1 mM $MgCl_2$ to the assay medium did not change the ATPase activity of either myosin or actomyosin, indicating that trace Mg concentrations do not influence these values. [b] The values cited for the fiber are for the following: "+actin", the ATPase activity at full filament overlap (2.0–2.2 μm) in Figure 2; "−actin", the ATPase activity extrapolated to zero filament overlap at 3.85 μm.

The data in Table I were obtained by mixing the myosin and the actin in high ionic strength and diluting to 150 mM KCl. If the myosin was first precipated in 150 mM KCl and actin (50 μM) was subsequently added and mixed by stirring, the K-ATPase activity was the same as that found above, 0.22 ± 0.025 s^{-1}. If the actomyosin was incubated with 50 μM Mg-ATP for 5 min in 150 mM KCl, producing superprecipitation, and EDTA was then added and the K-ATPase activity assayed, one again finds a low value, 0.21 s^{-1}. Thus, the K-ATPase activity of the actomyosin is independent of the method of preparing the actomyosin. The addition of up to 0.1 mM Mg to the assay mixture did not alter the ATPase activity of either myosin or actomyosin, showing that trace amounts of Mg were not influencing the results.

Rizzino et al. (1970) used the K-ATPase activity to determine the number of HMM molecules that would bind per actin. Because they found that one HMM bound per actin, in disagreement with current ideas of this interaction (Margossian & Lowey, 1978), we decided to reinvestigate this problem. In order to obtain additional information on the interaction, we determined the binding directly by sedimenting the actin–HMM complex. The results shown in Figure 2 demonstrate several points: (1) HMM and actin do form a complex in the presence of K-ATP, (2) the binding constant determined from this method (6×10^4 M^{-1}) is similar to that determined from the ATPase studies (10^5 M^{-1}), and (3) the stoichiometry is ~1 HMM/2 actin monomers, in agreement with our current concept that each HMM head has one binding site for actin. We do not understand why Rizzino et al. (1970) obtained a stoichiometry of 1:1. ATPase activity measurements of samples similar to those sedimented gave results that agreed with the sedimentation results. Thus, there does not appear to be any indication that significant HMM binds to actin in a 1:1 complex under the conditions of our assay.

The difference in ATPase activity between free myosin and myosin bound to actin can be used as a measure of the fraction of myosin heads not bound to actin in a glycerinated fiber. The

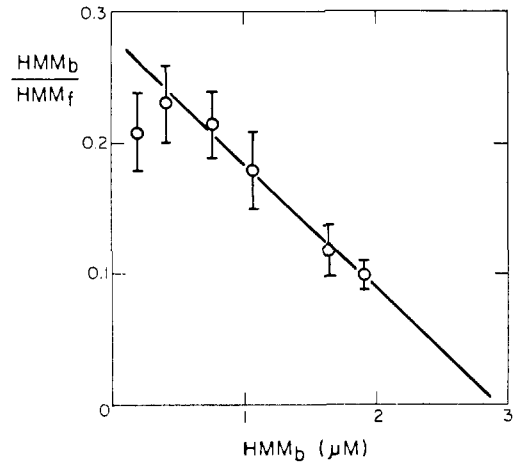

FIGURE 2: Scatchard plot of the binding of HMM to actin under the assay conditions given in Figure 1. Actin and HMM were mixed and rapidly sedimented in an air driven centrifuge. In the absence of HMM virtually all the actin sedimented under these conditions. Gel electrophoresis in the presence of $NaDodSO_4$ showed that all protein in the supernatants was HMM_f. The protein contents of both pellets and supernatants were then used to determine the amount of HMM that bound to actin (HMM_b). The actin concentration was 5 μM and HMM varied from 1 to 25 μM. The solid line represents a least-squares fit to the data after exclusion of the point at the lowest HMM_b.

ATPase activity of fibers is shown as a function of the sarcomere length in Figure 3. The sarcomere length was determined by laser diffraction both before and after the assay, and data from fibers that did not have sharp diffraction patterns at the end of the assay were discarded. Bundles of from 5 to 15 fibers were used. No dependence on bundle size was observed in this range, indicating that diffusion of ATP into the bundle is not a problem. At zero filament overlap the K-ATPase activity of the fiber is close to, and in fact a little higher than, that of myosin measured in solution. This demonstrates that free myosin heads in the fiber have a high K-ATPase activity and that the fact that the myosin is "in situ" in a fiber bundle does not impair our ability to measure its ATPase activity. The most important aspect of this figure is that in the range of sarcomere lengths from 2.0 to 2.2 μm, where there is complete overlap between actin and myosin filaments (Page & Huxley, 1963), the ATPase activity of the fiber is close to that measured for myosin bound to actin. In addition, the ratio of the K-ATPase activity at complete filament overlap to that at zero overlap (3.85 μm; Page & Huxley, 1963) is similar to the ratio of actomyosin K-ATPase activity to that of myosin measured in solution (see Table I). This indicates that all or most of the myosin heads are bound to actin in the region where the filaments overlap. As the sarcomere length increases, the overlap between filaments decreases and the ATPase activity increases because some myosin heads can no longer interact with actin filaments. The data approximately follow a linear dependence on sarcomere length above $\sim$2.2 μm in Figure 3. The data also clearly indicate that at shorter sarcomere lengths the myosin heads are unable to bind to actin, as expected from studies of fiber stiffness (Halpern & Moss, 1976).

The fraction of free heads can be calculated from the measured ATPase activities, V, as follows: fraction free = $(V_{\text{fiber}} - V_{\text{actin-myosin}})/(V_{\text{myosin}} - V_{\text{actin-myosin}})$. Using the data of Table I, we find that 6% of the myosin heads are free. For a function $f(X_n)$ of variables (assumed to be statistically independent) X_n, with standard errors of ΔX_n, the standard error, Δf, is estimated by $(\Delta f)^2 = \sum(\partial f/\partial X_n)^2 \Delta X_n^2$. This expression gives a standard error on the number of free heads of $\pm$4%.

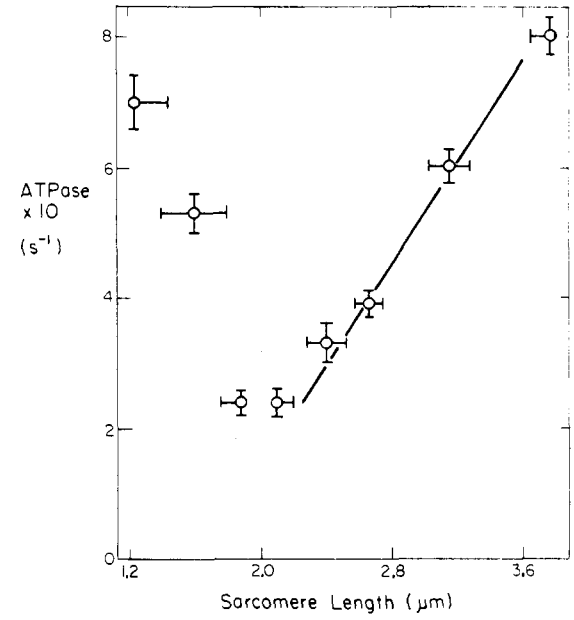

FIGURE 3: K-ATPase activity of glycerinated rabbit psoas fibers (s^{-1}/head) as a function of the sarcomere length. The assay conditions were the same as those described in Figure 1. The straight line was fit to the four data points at the longer sarcomere lengths. The error bars on the ATPase activity values represent the standard error of the mean for at least five measurements. The error bars on the sarcomere lengths represent the spread in sarcomere lengths as measured by diffraction.

Therefore, the values of the K-ATPase activities indicate that 94 $\pm$ 4% of the myosin heads are bound to actin in a rigor psoas muscle, in which there is complete filament overlap.

The above calculation assumes that one can calculate the fraction of bound heads in a fiber by using the K-ATPase activities measured for actin and myosin in suspensions. This calculation involves several assumptions, the most important and questionable one being that the K-ATPase activity of a bound head in a fiber is equal to that of a bound head in an actomyosin suspension. For instance, if one takes the value for acto-S-1 in place of that for actomyosin, the above calculation would predict that 17% of the heads remain unbound. Thus, the fraction of bound heads was determined by a separate method which relied on measurements of the ATPase activities as a function of the KCl concentration.

As the KCl concentration is decreased, the K-ATPase activity of myosin decreases dramatically (Seidel, 1969) while that of actomyosin or acto-S-1 is constant, as shown in Figure 4. Figure 4 also shows that the activity for a fiber (sarcomere length = 2.2 μm) is constant as a function of KCl and that a fiber stretched to zero overlap has a dependence on KCl that resembles myosin. Thus, the ATPase activity of any unbound myosin heads in the fiber decreases with decreasing [KCl], making the K-ATPase activity of the fiber also a function of [KCl]. The lack of dependence of the fiber K-ATPase activity on KCl concentration again shows that there are no unbound myosin heads in the fiber. This can be expressed mathematically by $\Delta V_{\text{fiber}} = f\Delta V_{\text{acto-S-1}} + (1 - f)\Delta V_{\text{myosin}}$, where ΔV is the change in K-ATPase activity on changing from 50 to 150 mM KCl and f is the fraction of attached heads. Substitution of values from Figure 4 into this equation gives f = 100 $\pm$ 5%. Thus, this calculation concludes that all the myosin heads are attached in the rigor fiber at full filament overlap.

We have investigated the K-ATPase activity of myosin and actin in the presence of ATP and the absence of divalent cations. To assess the effect of ATP on the actomyosin interaction under these conditions, we estimated the dissociation

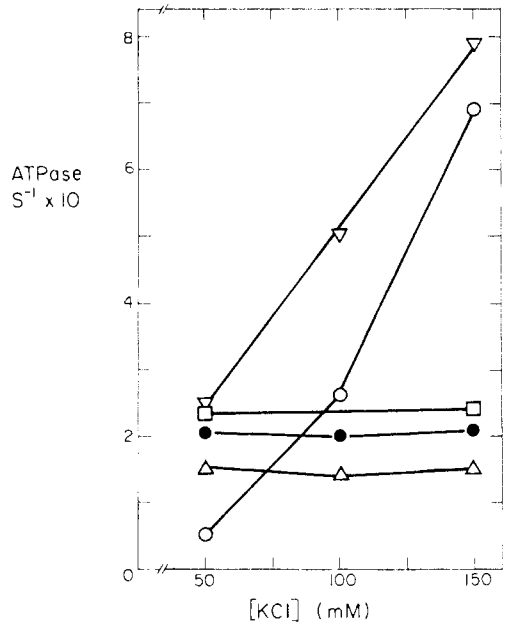

FIGURE 4: K-ATPase activity (s^{-1}/head as a function of the KCl concentration. The medium contained 4 mM EDTA, 4 mM ATP, 50 mM Tes, pH 7.5, and a variable amount of KCl. Samples consisted of myosin (○), actomyosin (●), acto-S-1 (△), and glycerinated psoas fibers with a sarcomere length of 2.2 (□) or 3.8 (▽) μm. Protein concentrations were 0.44 μM myosin or 1.2 μM S-1 and 50 μM actin. Glycerinated fibers were assayed as in Figure 2. Each point represents an average of more than six measurements, with standard errors of the mean of 0.01–0.02 s^{-1}.

of myosin from actin by ATP in the fiber by measuring the change in the rigor stiffness of the fiber on addition of ATP. The rigor stiffness was determined by measuring the change in length that followed a change in tension. Upon addition of 4 mM ATP to a fiber in 150 mM KCl, 4 mM EDTA, and 20 mM Tes, pH 7.5, the rigor stiffness did not change from the control value within experimental error (±10%). Thus, the presence of ATP does not cause significant dissociation of myosin from actin in the fiber. Under these conditions ATP did not cause the fiber to generate an active tension. This suggests that myosin, actin, and ATP form a ternary complex in the absence of divalent cations and that the actin–myosin complex splits ATP at a slower rate than does myosin by itself. If this is the correct interpretation, the apparent binding constants obtained from the double-reciprocal plots or from the binding studies indicate that the strength of the actin–myosin interaction is decreased by about an order of magnitude in the presence of K-ATP. We note parenthetically that the apparent binding constants to actin for myosin, HMM, and S-1 were all approximately the same (within a factor of 10), again apparently implying that the two heads of the double-headed molecules bind to actin with negative cooperativity (Highsmith, 1978; Margossian & Lowey, 1978).

Discussion

The ATPase activity of myosin or actomyosin is altered severely by the absence of Mg, as discovered by Hasselbach (1957) and studied in more detail by Rizzino et al. (1970). Our results agree with those of Rizzino et al. (1970) that addition of actin inhibits the K-ATPase activity of myosin but does not abolish it completely. However, they achieved a greater degree of inhibition than was found here, 90% vs. 70%, and we found a different stoichiometry for the binding of HMM and actin.

Two lines of evidence, the direct binding studies and the measurements of fiber stiffness, both indicate that under the assay conditions employed here ATP does not cause measurable dissociation of myosin from actin. Thus, although a transient dissociated state may be part of the ATPase cycle, the two proteins remain bound throughout most of the cycle. In spite of the fact that the details of the ATPase mechanism are not known, the inhibition of the myosin K-ATPase activity by actin can be used as a probe of the fraction of myosin interacting with actin in a given situation. An additional probe is provided by the fact that the K-ATPases of myosin and actomyosin have distinctly different dependences on the KCl concentration.

We first address the question of the fraction of myosin heads bound to actin in a suspension of actomyosin. Each myosin head has a high affinity binding site for actin, and thus in the presence of excess actin one might expect that all heads would be bound to actin. However, the ability of the myosin heads to interact with actin could be restricted by the presence of the tail which joins the two heads or by the aggregation of the tails. If some heads were free in the actomyosin suspension, it would account for the fact that the K-ATPase activity of the actomyosin is slightly greater than that of acto-S-1 where no restrictions on interaction are present. However, we feel that this is an unlikely interpretation since the presence of free myosin heads would make the actomyosin ATPase activity depend on KCl concentration. Thus, we conclude that all myosin heads are bound to actin at high actin concentrations and that the difference between actomyosin and acto-S-1 K-ATPase activities could be due to some difference in the interaction in the two cases.

The above conclusion has important implications for the interpretation of the actomyosin interaction in the presence of Mg-ATP. The Mg-ATPase activity of actomyosin at saturating actin is ~0.5 s^{-1}, which is much lower than the maximum activity of acto-S-1 (~20 s^{-1}). This difference has sometimes been attributed to a steric blocking effect in which some myosin heads cannot interact with actin in an actomyosin mixture. However, our results show that most heads do interact with actin to at least the extent required to inhibit K-ATPase. Therefore, we feel that it is unlikely that the Mg-ATPase activity of actomyosin is low because myosin heads do not interact with actin, and thus this difference in activities must be due to some difference in the manner in which myosin and S-1 interact with actin.

This conclusion is consistent with current models of muscle contraction which have a slow rate of detachment of heads over some region that produces force and a fast rate of detachment near the end of this region (Huxley, 1957; Cooke & Bialek, 1979; Hill et al., 1975). Such models predict that acto-S-1 would have a greater ATPase activity than actomyosin if the S-1 is more free to change configuration while bound to actin than is myosin.

The data show that the K-ATPase activity of the fiber at complete filament overlap is close to that of myosin interacting with excess actin in suspension, and its dependence on [KCl] again resembles that of actomyosin, while the K-ATPase activities of a fiber at zero filament overlap resemble those of myosin alone in solution. These results all fit into a simple picture. The K-ATPases of a free myosin head in a fiber are similar to those of a myosin head in solution, and at full filament overlap all myosin heads can interact with actin. Thus, these results show that the incorporation of myosin and actin into the organized filament array of the fiber has not impaired its ability to interact with actin under the conditions employed here. Despite mismatch between subunit repeats of actin and myosin filaments and steric difficulties introduced

by the helical nature of both filaments, all myosin heads in the fiber can find actins with which they can interact. This result implies that a large degree of flexibility must exist in the myosin bond and possibly also in the actomyosin bond.

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Cell-Free Synthesis of Cartilage Proteins: Partial Identification of Proteoglycan Core and Link Proteins†

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ABSTRACT: A poly(adenylic acid)-enriched RNA fraction isolated from calf articular cartilage was translated in cartilage and wheat germ cell-free systems. The radioactive translation products were assayed for the presence of two cartilage proteins: proteoglycan core and glycoprotein link. This was accomplished by utilizing the property both proteins have of binding to hyaluronic acid and forming an aggregate large enough to elute in the void volume of a Sepharose column. When an extract of calf cartilage, containing hyaluronic acid and link, was added to the cell-free mRNA directed products synthesized in a cartilage system and applied to a Sepharose 6B column, 5–10% of the radioactive material was recovered in the void volume of the column. Analysis of the radioactive material in this excluded fraction after separation by electrophoresis on sodium dodecyl sulfate–polyacrylamide gels revealed five major radioactive bands with the following molecular weights: >300 000, 51 000, 47 000, 41 000, and 28 000. Similar results were obtained with cartilage mRNA directed synthesis in a wheat germ system. The largest protein migrates with a molecular weight described by many for proteoglycan core. The four lower bands have molecular weights similar to those described for proteoglycan links. The 41 000 and 28 000 proteins form a precipitate with antibody to homogeneous link.

Articular cartilage consists of chondrocytes dispersed in a hyaline matrix formed by type II collagen and at least two glycosylated proteins noncovalently bound to hyaluronic acid (Rosenberg, 1978; Mankin, 1970; Hascall, 1977). One of the glycoproteins (proteoglycan link) of molecular weight 45 000–50 000 is believed to stabilize the interaction between hyaluronic acid and the second glycoprotein, referred to here as proteoglycan subunit [M_r (0.5–4.0) × 10^6] (Bonnet et al., 1978; Baker & Caterson, 1978, 1977; Keiser, 1975; Oegema et al., 1977; Caterson & Baker, 1977; Swann et al., 1976). The protein part of the subunit, known as core [M_r (1.8–2.0) × 10^5], constitutes only 10% of the proteoglycan with the bulk of the molecule consisting of the sulfated glycosaminoglycans chondroitin sulfate and keratan sulfate. The chondroitin sulfate chains are covalently bound to serine and threonine residues of core and account for the majority of the molecule's polysaccharide component. The remaining carbohydrate is keratan sulfate bound to one of three amino acids: glutamic acid, serine, or threonine (Sweet et al., 1978; Brandt et al., 1973; Bayliss & Ali, 1978; Hardingham & Muir, 1972).

Core protein is considered by many to contain three distinct regions: a hyaluronate binding area [M_r (6–7) × 10^4] of constant amino acid composition, an area adjacent to this which is rich in keratan sulfate, and a polysaccharide attachment region of variable length and amino acid composition to which is bound the majority of chondroitin sulfate chains (Serafini-Fracassini & Smith, 1974). There is evidence that the hyaluronate binding area of the proteoglycan contains residues that form an ionic bond with the carboxyl groups of hyaluronic acid (Hardingham et al., 1976; Lindahl & Hook,

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