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AN ACTIVATION MECHANISM FOR ATP CLEAVAGE IN MUSCLE

William F. Harrington

Department of Biology and McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland

Emil Reisler

Department of Polymer Science, Weizmann Institute of Science, Rehovot, Israel

Morris Burke

Department of Physiology, University of Maryland School of Dentistry, Baltimore

Evidence for a proposed activation mechanism is summarized. The low rate of ATP cleavage in the resting state of muscle is considered to result from the formation of a stable ring structure involving the two essential sulfhydryl groups on each myosin head and MgATP. Activation is thought to occur by interaction of actin in the vicinity of one of the essential sulfhydryl groups, Thus opening the stable ring leadin to rapid dissociation of split products. This idea is consistent with the kinetic scheme of ATP cleavage developed recently by other workers and allows a prediction of the shift in population of intermediate states with changes in solvent conditions. It is also supported by our recent studies on the spatial geometry of the ring. The possibility that other nucleophilic groups may replace the sulfhydryl groups in other contractile systems is considered. The relevance of the ring structure to the tension generating event is discussed on the basis of recent measurements of the rate of contraction of modified (SH_1-blocked) actomyosin threads. Results indicate that the ability to form the ring structure is an essential requirement of the contractile process in these systems, and, moreover, that single, modified heads of myosin can act independently to produce the same rate of contraction as native myosin. This latter finding suggests that the myosin duplex exhibits some type of negative cooperativity in the contractile process.

INTRODUCTION

It is now well established that the basic element of vertebrate muscle, the sarcomere, consists of thick and thin filaments arranged in space in a double hexagonal lattice, and that muscle contraction involves a process in which the thin filaments of the array slide past the thick filaments. According to low-angle X-ray studies of living, resting muscle (1), crossbridges, which are known to be constructed from the globular heads of individual myosin molecules, are arranged in a helical pattern along the surface of the thick filaments. In the resting state the filaments are bathed in an ionic environment of millimolar MgATP, low ionic strength (~0.15 M) and very low Ca^{2+} (~ 10^{-8} M). When muscle is activated, the Ca^{2+} level rises abruptly (in a few milliseconds) to about 10^{-5} M, the bridges move out to the thin filaments, and the original integral helix of crossbridges disappears from the X-ray diffraction pattern. At the present time we do not know precisely how this major translocation of mass is triggered or controlled. The most recent evidence (2) indicates that about 40–50% of the crossbridges in vertebrate muscle instantaneously

attach to the actin filaments during active contraction. Moreover, it is now generally believed that activation of vertebrate striated muscle is initiated within the thin filament complex (actin-tropomyosin-troponin) through binding of Ca^{2+} to the troponin-C component of this system (3, 4). Additionally, there are recent indications that some type of Ca-dependent structural transition may also occur within the thick filaments during the initial stages of contraction (5–7).

In the absence of calcium, the regulatory proteins inhibit association of the crossbridge with actin. On activation, the elevation in Ca^{2+} concentration saturates the Ca^{2+} binding sites on the troponin complex and this process in turn allows actin-myosin interaction and subsequent development of tension. Since there is only one troponin molecule for every seven actin molecules (8), the control of activation is presumably mediated by tropomyosin molecules which form continuous threads along the grooves of the actin double helix (9, 10).

It is clear from a large number of in vitro experiments that combination of the crossbridge with a special binding site (5) on the actin helix is responsible for a very rapid increase in ATP hydrolysis, but the mechanism of this activation is unknown. We do know that the ATP hydrolysis rate of purified myosin, that is, myosin free of actin, is strongly inhibited in the presence of millimolar MgATP even at high concentrations of Ca^{2+}. How is it, then, that in muscle, which has a virtually invariant level of MgATP, the cleavage reaction is so strongly accelerated simply by association of the crossbridge with the actin filament? A partial answer to this question has been provided by recent kinetic studies of ATP cleavage by myosin and actomyosin (11, 12). It appears from this work that the decay of a rate-limiting, long-lived, intermediate species, formed in the reaction sequence of ATP hydrolysis, is markedly accelerated through association of the crossbridge with actin. But the question still remains as to how simple association of the two proteins, myosin and actin, could lead to this accelerating effect which is clearly essential in providing the energy required for contraction. To answer this question, we must ultimately understand why Mg^{2+} inhibits ATP cleavage in an actin-free system and why, in the absence of Mg^{2+}, the complementary divalent metal Ca^{2+} strongly accelerates the cleavage rate. Additionally, we would like to know if these phenomena have special significance in understanding the tension-generating event itself. The purpose of this paper is to summarize recent studies in our laboratory which are relevant to these questions.

EVIDENCE THAT MgATP FORMS A STABLE RING STRUCTURE INVOLVING THE TWO ESSENTIAL SULFHYDRYL GROUPS

Chemical modification studies offer a powerful tool to investigate enzymatic mechanisms and we have employed this approach in our attempts to gain insight into the structural reasons for the striking inhibition of ATP cleavage by myosin in the presence of Mg^{2+}. In our approach to this problem we have been strongly influenced by the earlier work of Sekine and Kielley (13, 14) demonstrating the importance of two particular sulfhydryl groups (identified as SH_1 and SH_2) to the enzymatic properties of myosin. This pair of essential sulfhydryl groups is located within each

globular head of the duplex myosin structure and the blockage of one sulfhydryl group (SH_1) per subunit elevates the Ca^{2+} ATPase activity about four-fold while causing a concomitant loss in the EDTA-ATPase activity and in the ability of actin to activate (13, 15). Subsequent blocking of a second thiol group (SH_2) results in complete loss in myosin ATPase activity (14). Chemical blocking of these two groups (by N-ethyl maleimide or fluorodinitrobenzene) can be carried out with a high degree of selectivity (13, 14, 16) and can be linearly related to changes in activity as shown by isolation and identification of sulfhydryl peptides.

In our studies (17), attempts to react the two SH groups with a blocking reagent (NEM) in the presence of millimolar MgATP (Fig. 1A) revealed that the two groups are simultaneously protected. It will be seen that virtually full protection of the SH_1 group to modification by the blocking reagent occurs (Ca-ATPase is invariant with time of reaction) and strong protection is also afforded the SH_2 group (EDTA-ATPase levels off at a relatively high value). This feature is unique to MgATP and could not be duplicated by any other substrate examined including MgADP, CaATP, CaADP, Mg^{2+}, or free nucleotide. As can be seen in Fig. 1B the time-dependence of the modification reaction is unaffected by the presence of these substrates. Since only one MgATP is bound per individual head (SF_1), this information strongly suggestes that some type of cooperative interaction is occurring between the MgATP substrate and the two essential sulfhydryl groups. On the basis of such studies we have proposed that a ring type structure is formed. The formation of such a closed ring structure could account for the unique inhibition displayed by MgATP in relation to other divalent metal nucleotides.

Assuming that such a quasistable ring structure is responsible for the inhibition observed, then a process which would open the ring or prevent its formation would be expected to abolish the inhibiting effect of Mg^{2+} resulting in a marked activation of myosin ATPase. Activation of myosin ATPase in the presence of Mg^{2+} has been noted in earlier studies (13) following chemical modification of the SH_1 site. Such activation can be viewed as resulting from the inability of a chemically-blocked SH_1 site to participate in the formation of the stable ring structure. Could actin act in an analogous manner? That is, could actin, by associating with the myosin crossbridge at or near the SH_1 site, accelerate the cleavage reaction by breaking the inhibiting ring. This possibility is certainly in accord with the activation profile shown in Fig.2 where the relative ATP cleavage activity vs. p Mg^{2+} is compared for actomyosin and SH_1 blocked myosin (no actin). It will be observed that the two systems are indistinguishable and both show maximum activation in the physiological concentration range of MgATP. It is also in accord with the shift in the EPR spectrum of SH_1 spin-labeled myosin on interaction with actin (18, 19).

The fact that we assign such a specific effect to actin is consistent with the well-known experimental observation that purified actin (free of the regulatory proteins troponin and tropomyosin) does not exert an accelerating effect on myosin ATPase in the presence of Ca^{2+} (20). This is not unreasonable in light of X-ray crystallographic evidence, indicating that CaPPi and MgPPi differ in their spatial geometries in that the terminal phosphate, thought to be analogous to ATP in chelation with the metal, is staggered in the case of Ca^{2+} and eclipsed in the case

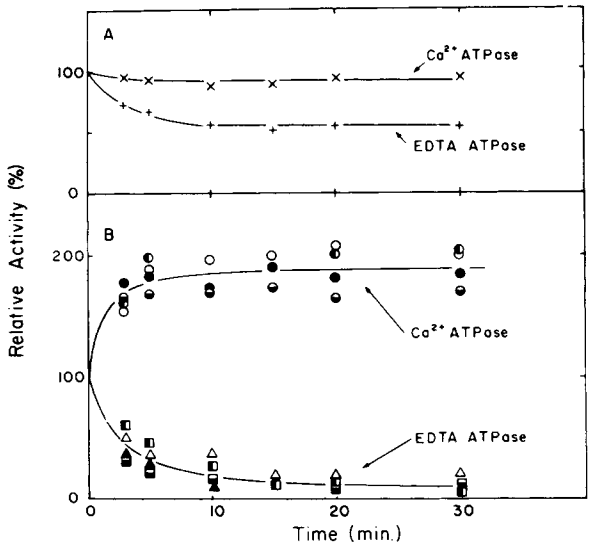


Fig. 1. Relative ATPase activity of myosin reacted with a four-fold molar excess of NEM over myosin as a function of time of modification. A. In the presence of 10^{-3} M MgATP. B. In the presence of 10^{-3} M solutions of the following ligands: ▲, ●-ADP; △, O-$MgCl_2$; ▪, ◒ CaADP; □, ○ MgAMP. For further experimental details see Reisler et al. (17).

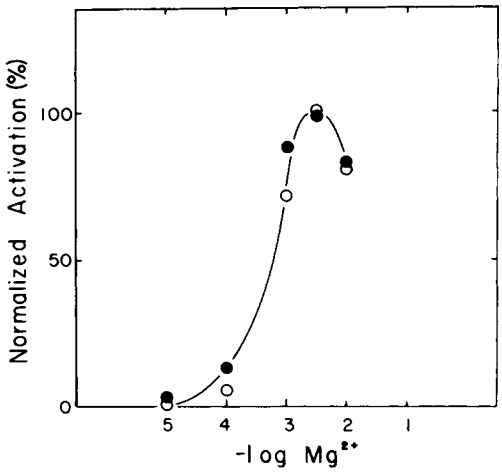


Fig. 2. Normalized effect of added Mg^{2+} on activation of ATPase activity of myosin by actin (●) or by modification of the SH_1 sties with NEM (○). (From Reisler et al. (29)).

of Mg^{2+} (21–23). Thus, the difference in chelation geometry may account for the inability of Ca-ATP to form a stable ring on binding to the myosin crossbridge.

RELATIONSHIP OF THE RING STRUCTURE TO THE KINETIC SCHEME OF ATP CLEAVAGE

A kinetic scheme for ATP cleavage by myosin in the presence of Mg^{2+} has recently been developed through the work of several laboratories (11, 12, 24). The scheme in its simplest form may be stated as follows:

1. M + MgATP $\rightleftharpoons$ M*MgATP
2. M*MgATP $\rightleftharpoons$ M**MgADP·Pi
3. M**MgADP·Pi $\rightleftharpoons$ M*MgADP·Pi
4. M*MgADP·Pi $\longrightarrow$ Products.

The essential feature of the scheme is the formation of a long-lived intermediate species (M**MgADP·Pi) following bond hydrolysis. Under physiological conditions of ionic strength and temperature, the decay rate of this species is the rate-limiting step for the overall hydrolysis reaction. This step is thus responsible for the marked inhibition of ATP cleavage in the presence of mM concentration of Mg^{2+}. We identify this intermediate with the stable ring structure discussed above. Consequently, we assume that whenever MgATP combines with a myosin head the system will exist predominantly in the ring structure and the participating SH_1 and SH_2 sites will therefore be protected from chemical modification by thiol reagents. This will not be the case in the presence of MgADP which, on combination with SF_1, is believed to form an intermediate species in the reaction pathway (25) subsequent to decay of the long-lived intermediate. This complex has been identified as the Michaelis-Menton complex and, as we have discussed earlier, does not exhibit a protective effect to chemical modification of the SH_1 and SH_2 sites. Thus, we have a very useful and simple method to establish the presence of the long-lived intermediate by comparison of rates of modification of SH groups in the presence of MgADP and MgATP.

According to the Lymn-Taylor view, actin enters the crossbridge cycle at the long-lived intermediate step and increases the decay rate of this intermediate, thus accelerating the rate of flux through the hydrolytic cycle. In terms of our proposal that the ring structure can be equated with the long-lived intermediate, we suggest that the activating effect of actin stems from its ability to open the ring and allow rapid release of the split products from the SH_2 site. Turning to the scheme depicted in Fig. 3, we correlate the four major steps of the cleavage cycle with the formation and opening of the cyclic structure. The cycle starts with the myosin crossbridge (SF_1) associated with the actin filament. Binding of MgATP dissociates the actomyosin complex, allowing the cyclic ring to be formed on the free myosin head (here we have omitted the isomerization step following the formation of the binary SF_1-MgATP complex deduced recently by Bagshaw et al (12) from stopped-flow studies). Following bond cleavage the split products remain locked into the stable, long-lived ring structure which, however, can now recombine with actin, presumably as a result of the

reduction in competitive affinity of the substrate for the SH_1 site. This step opens the ring, resulting in rapid dissociation of the split products from the weaker binding SH_2 site (26).

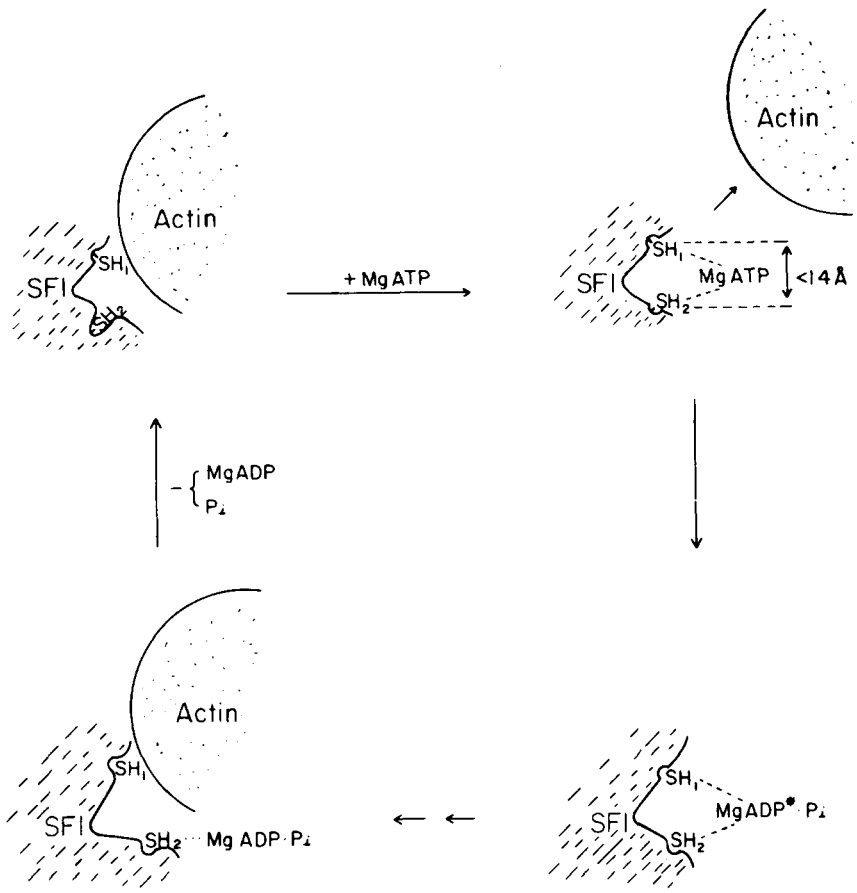
The stability of the ring structure suggests that it would be difficult to modify either of the essential SH groups while the long-lived intermediate state exists. Indeed, we have seen that MgATP is the only substrate which offers protection to blocking of these groups. Thus we would expect that in the presence of actin, where the intermediate species is no longer rate-limiting, such protection should be reduced. In Fig. 4 we see that this is indeed the case. When we follow the loss of EDTA-activated ATPase activity on modification with NEM, we observe little protection by MgATP in the presence of actin, whereas a strong protective effect is seen in the absence of actin. These studies were carried out at physiological ionic strength (0.15 M KCl) and 25°C, but if the same experiment is repeated under conditions minimizing actomyosin interaction (0.5 M KCl) then the life-span of the long-lived intermediate is not affected by actin and we observe strong protection. Conversely, at very low ionic strengths (0.05 M KCl) and 25°C where actomyosin interaction is strong, just the reverse is observed.

Several workers (24, 12, 27) have demonstrated a shift in the relative populations of the long-lived intermediate and the succeeding Michaelis-Menton complex, as a consequence of changes in the decay constant of the latter species with temperature and ionic strength. According to our view, these changes should be reflected in the relative populations of the stable ring structure, which in turn can be monitored through changes in the protective effect of the structure to modification of the two essential thiol groups. Lowering the ionic strength as well as lowering the temperature decreases dramatically the rate of decay of the Michaelis-Menton complex, thus acting to increase the population of this complex with respect to the long-lived (ring) structure (see Table I). In fact at low temperature (5°C) and low ionic strengths (0.05 M), the Michaelis-Menton complex is the predominating species.

TABLE I. Population of Ring State Structure (%) Under Various Ionic Strength and Temperature Conditions*

	Ionic Strength					
	0.05		0.15		0.5	
Protein	5°	25°	5°	25°	5°	25°
Subfragment I	37	67	56	81	73	86
Myosin	–	–	–	–	74	–
Native actomyosin	0	0	38	43	74	84

*Estimation based on protection afforded by MgATP to NEM modification of active sites.



Proposed Mechanism for Actin Activation of the Myosin Cross Bridges

Fig. 3. Schematized description of the crossbridge cycle in relation to the formation and breaking of the stable cyclic intermediate.

Our reason for stressing this point is to emphasize that studies designed to determine the mechanism of ATP hydrolysis by actomyosin should be carried out under conditions which overwhelmingly favor the long-lived intermediate, since the activating effect of actin is now believed to arise directly through its effect on this intermediate state. We find, for example, that chemical blocking of the SH_1 site yields the same activation of ATP cleavage at physiological ionic strengths and at 25°C as that achieved by actin, whereas at much lower ionic strengths and temperatures this correspondence is no longer observed (28).

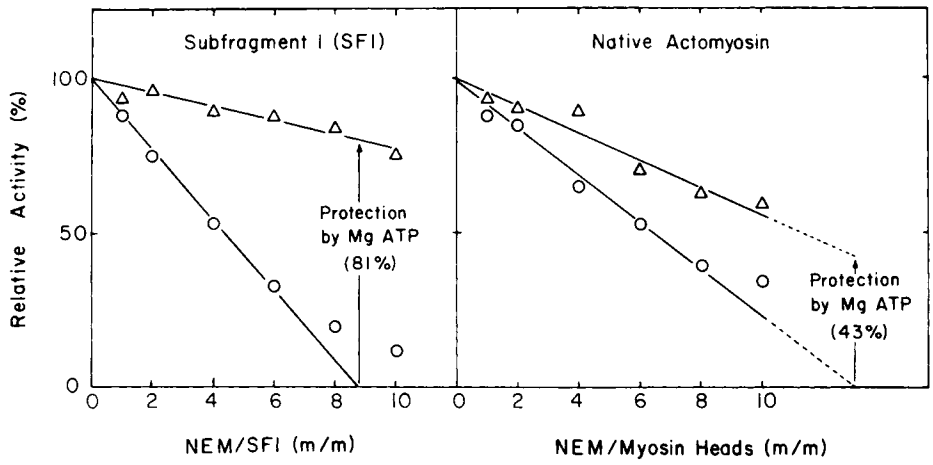


Fig. 4. Loss of EDTA ATPase activity of actomyosin or of subfragment I (no actin) by modification with NEM in the presence of millimolar MgATP or MgADP. Reaction with NEM was allowed to proceed for 5 min at 25°C in 0.15 M KCl, pH 7.9.

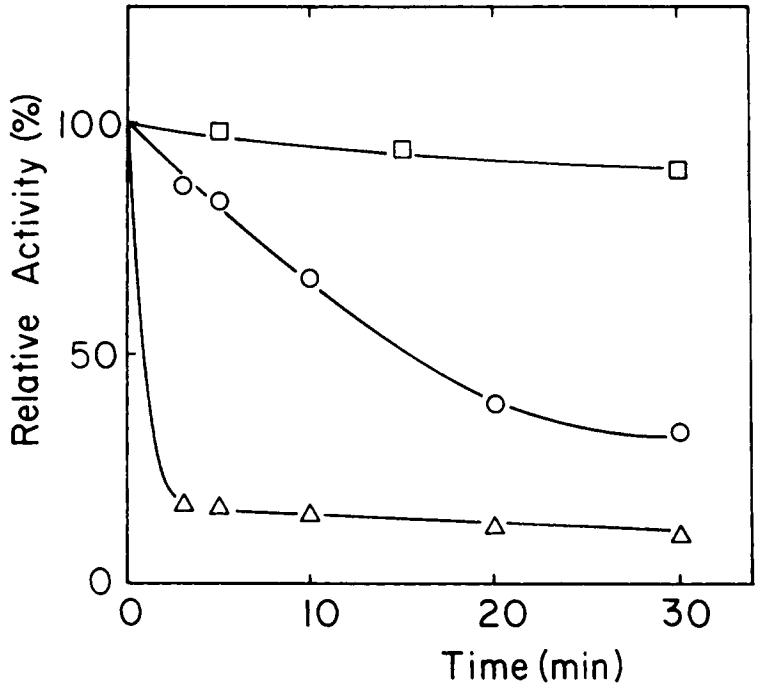


Fig. 5. The effect of pH and substrate on the rate of ring closure of SH_1-p-phenylenedimaleimide modified myosin. Experiments were done at pH 7.0 (□); pH 7.9 (○); and pH 7.9, 1 mM MgADP (△). (From Reisler et al. (29)).

SPATIAL GEOMETRY OF THE RING STRUCTURE

It will be clear that formation of the ring structure imposes considerable spatial restraints on the location of the two essential SH groups since these groups must be located (at least transiently) at a distance which would allow bridging with the MgATP substrate. We have investigated the spacing between the essential thiol groups through the use of bifunctional maleimide reagents. Among the reagents used, p-phenylene dimaleimide displays the closest similarity to NEM in its reactivity and specificity for the essential SH groups of myosin. We have found that this reagent has an appropriate three-dimensional geometry to link covalently the SH_1 and SH_2 groups with a high degree of specificity. From an examination of space-filling models of this covalent bridge, we deduce that the two essential thiol groups may reside 12–14 Å apart on each SF-1 molecule (29).

Figure 5 shows closure of the ring by the bifunctional reagent, one arm of which has been initially and preferentially attached to the SH_1 group. After removal of the excess of the reagent on Sephadex columns, closure through reaction with the SH_2 group occurs very rapidly on addition of MgADP (1 mM). The rate of this process is completely independent of protein concentration as might be expected for an intramolecular reaction. It is of considerable interest that the closure reaction (at pH 7) requires the presence of MgADP. Earlier studies (14) have demonstrated that preferential blocking of the SH_2 group by the monofunctional thiol reagent, NEM, can occur in the presence of this substrate suggesting that the SH_2 group is normally inaccessible to attack, but is unmasked or "popped out" when MgADP (or MgATP) is bound in the active site region.

DO SH GROUPS PLAY A UNIQUE ROLE IN FORMATION OF THE STABLE RING?

Up to this point we have considered the stable ring to involve participation of two specific thiol groups in each myosin head. Are sulfhydryl groups essential to the type of structure which we envision for the long-lived, rate-limiting intermediate in the ATP cleavage sequence, or is it possible to replace the nucleophilic sulfhydryl group with another type of nucleophilic group, say an amino group? If this were true, the ring structure could have a more general significance. Conceivably, such structures could play an important role in the reaction sequence of ATP cleavage in other contractile systems which are either devoid of cysteine or in which thiol groups lack the appropriate spatial geometry to form the ring. We have approached this question (17) through a two-step modification of the SH_1 groups of myosin. In the first step, this group was blocked with fluorodinitrobenzene, thus eliminating the possibility of ring formation with MgATP. The properties of the modified myosin were similar to those observed following NEM-SH_1 modification (i.e. elevation of Ca^{2+} and elimination of EDTA-ATPase activity). The nitro groups of the covalently linked dinitrophenyl derivative were subsequently reduced to amino groups with dithionite. It will be seen from Fig. 6 that this modification, which creates a new nucleophilic center in the active site cleft, restores the ability of MgATP to form an inhibiting ring structure and the resulting profile of relative

ATPase activity vs p Mg^{2+} resembles that of native, unmodified myosin. That no major changes occur otherwise around the active center of myosin on reduction of SH_1-DNP to SH_1-phenylenediamine myosin is evidenced by the fact that the Ca-ATPase activity of the latter remains highly activated.

RELEVANCE OF THE RING TO THE TENSION GENERATING EVENT

Several workers (30 –33) have reported that tension generation in muscle requires the presence of Mg^{2+}. When Mg^{2+} is removed from glycerinated muscle fibers or myofibrillar preparations these systems are incapable of shortening or producing tension even though rapid ATP cleavage is observed in the presence of Ca^{2+}. These results suggest that tension generation in muscle may be directly linked to the ability of the myosin crossbridge to form a stable ring structure with MgATP. To probe this question we have attempted (34) to control the formation of the ring by selectively modifying the SH_1 site in glycerinated fibers as well as in homogenized myofibrillar preparations of rabbit skeletal muscle. In these studies it was essential to relate the contractile properties of the modified systems to the degree of SH_1 modification. However, we have been unable so far to find conditions for chemical modification such that the modified myosin isolated from these systems is selectively blocked at the SH_1 site. Consequently we could not unequivocally correlate the change in tension generating properties of such fibers with the presence of the stable ring structure. This intrinsic difficulty could, however, be overcome by employing native actomyosin preparations. In this case, selective modification of the SH_1 groups can be accomplished readily in the presence of mM Mg pyrophosphate where the actomyosin complex is present in the dissociated state. Following modification, the MgPPi is removed by dialysis and the resulting SH_1-blocked actomyosin aggregate is extruded into dilute salt to form conventional actomyosin threads. The rate of contraction of the threads in the presence of MgATP (5 mM) was determined by photographing these filaments as a function of time. Results are summarized in Fig. 7. We find very little change in the rate of contraction as a function of the degree of SH_1 modification (as measured by EDTA-ATPase of the isolated, SH_1-blocked myosin) until a major fraction of myosin heads are modified. At this stage further modification produces a rapid drop in the rate of shortening. Our results suggest that individual myosin heads are modified at random in the dispersed actomyosin system and that a myosin molecule with either head modified can participate in the contractile process. When the SH_1 groups of both heads of a single myosin molecule are modified, that molecule can no longer participate in the tension generating event even though hydrolysis of MgATP is proceeding at a rapid rate. This interpretation is consistent with the two plots presented in Fig. 7. The profile α is a theoretical curve showing the gradual increase in the fraction of myosin molecules with both heads modified as a function of the total fraction of modified heads. The solid curve, denoted $(1-\alpha)$ represents the loss of this species from the system. Or conversely, this theoretical curve can be viewed at each stage of modification as the sum of the two remaining species: (1) totally unmodified myosins, and (2) molecules with one modified head. It will be seen that the experimental

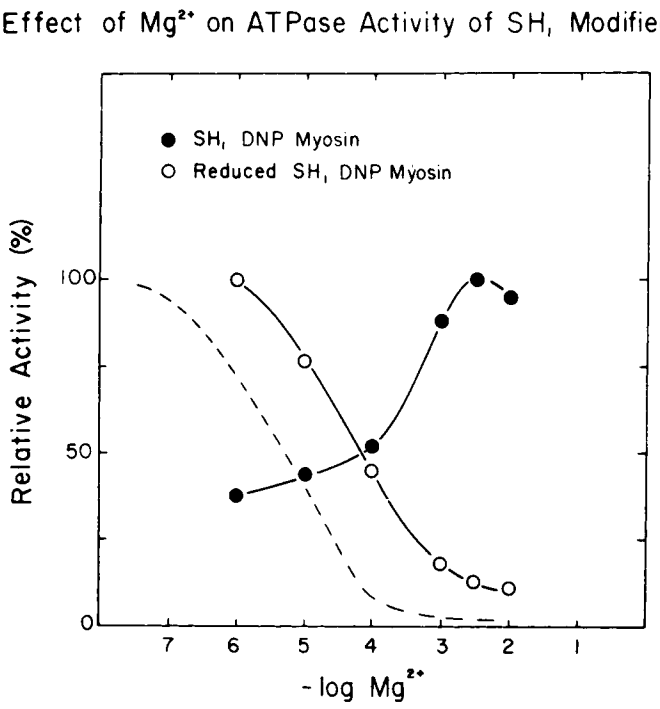


Fig. 6. Relative ATPase activity of SH_1-DNP modified myosin (•) and reduced SH_1-DNP modified myosin (○) as a function of the concentration of added Mg^{2+}. The dashed line shows the response of native myosin. (From Reisler et al. (17)).

points, which represent the normalized initial velocity of shortening, follow this theoretical profile very closely.

Two important conclusions stem from these results: (1) the ability to form the stable ring structure is an essential requirement of the contractile process; and (2) single, unmodified heads of myosin molecules (either head) can apparently act independently to produce the same rate of contraction as that of native myosin. This finding suggests that some type of negative cooperativity exists in the duplex myosin molecule during its interaction with actin. This latter conclusion would be consistent with the observations of Eisenberg and Kielley (20) that only one of the two myosin heads is bound to actin at any instant in in vitro actomyosin hydrolysis experiments.

CONCLUSION

To conclude this very brief review, we suggest that the long-lived intermediate (M**MgADP·Pi) in the ATP cleavage sequence can be identified with a cyclic structure formed between MgATP and two specific sulfhydryl groups. It seems possible that in other contractile systems alternative nucleophilic sites may function in an analogous manner. The stable ring has been utilized by nature to store the ATP

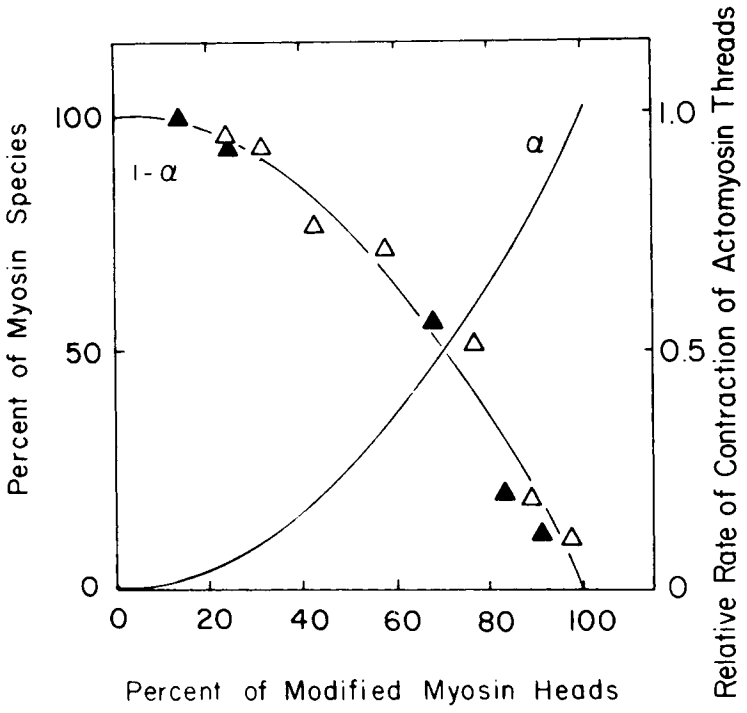


Fig. 7. Relative rate of contraction of actomyosin threads as a function of the extent of modification of myosin heads. The open and filled symbols represent experiments on different actomyosin preparations. The smooth curves represent (theoretical) populations of different myosin species present at any extent of modification – the curve α represents myosin molecules with both heads modified, while the curve (1-α) represents unmodified myosin plus myosin molecules modified at only one head.

cleavage energy over prolonged time periods, thus allowing minimal loss of energy in the resting state of muscle. Accordingly, we view the cyclic association of the crossbridge with actin as a process whereby the stable ring is opened through binding at or near one of the nucleophilic sites (SH_1) participating in the ring structure, thus accelerating energy conversion. Chemical modification of the SH_1 site in actomyosin systems, a procedure which prevents formation of the ring, also results in elimination of isotonic contraction, even though ATP cleavage is unimpaired, suggesting a direct linkage between opening of the ring and the tension-generating event. This possibility would be in line with the proposals of other workers that the decay of the long-lived intermediate may be directly coupled to the mechanochemical process which generates tension. Finally, our studies on the relationship between the contractile velocity of actomyosin threads and SH_1 modification focus again on an intriguing and long-standing enigma: if one head on each myosin molecule is sufficient to maintain contraction, what is the fundamental functional significance of the duplex structure of a myosin molecule?

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