Actomyosin Adenosine Triphosphatase Regulation by Intramolecular Myosin Mechanisms. Myosin Light Chains Functions and Rod Modification Effects

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Abstract. Mechanisms of the actomyosin ATPase modulation via the myosin light chains (LC) in various myosin types are discussed. The essential LC increase the stability of the myosin heavy chains (HC) in the myosin heads and, under certain conditions, they can affect the degree of interaction of HC with actin. The regulatory LC (RLC) are sensitive to calcium binding on specific sites or to calcium activated phosphorylation. These factors induce changes of the RLC state followed by changes of the HC state in response to calcium concentration changes during the contractile process. Direct calcium binding or phosphorylation effects in various muscles are mediated by special types of RLC and HC. Several examples of actomyosin ATPase changes induced by modifications of the myosin rod are compared. A common feature of these effects is a possible involvement of certain configurational changes of the myosin molecule. These changes can affect the spatial position of the myosin heads and the myosin-actin interaction.

Key words: Actomyosin ATP — Myosin light chains — Myosin heavy chains — Configurational changes

Introduction

In studies on myosin attention has primarily been paid to conformational transitions in the globular parts of the molecule (M-heads); these transitions govern the course of actomyosin ATPase reaction and play a leading role in its regulation. We shall discuss some of the myosin-linked regulatory mechanisms along with certain ATPase modification effects which cannot solely be accounted for by the properties of M-heads. These effects may represent manifestations of some yet unexplored mechanisms involving the myosin rod. We do not intend to give full information about myosin-associated control in this review; rather our purpose is to give an impulse to considerations on possible mechanisms of these regulatory processes.

All the myosin molecules in various muscles have identical quaternary structures. Two heavy chains (HC) (~200,000—210,000 daltons) form a long (~140 nm) rod and two globular parts, or „heads", each belonging to a single HC. In addition to HC, each head includes two non-identical subunits termed light chains (LC), differing in their amino acid composition and properties. One of these two LCs, which is more firmly bound, is essential for ATPase activity; we shall term it "ELC". In fast skeletal muscle there are two types of ELCs (~21,000 and 17,000 daltons, respectively); all other muscle myosins contain only one type of ELC. The other subunit in each M-head belongs to another, regulatory class (RLC) (19,000—20,000 daltons); RLC bind calcium ions.

Myosin affinity for nucleotides and for actin (A) as well as the actomyosin ATPase (AM ATPase) rate depend on the state of HC which form binding sites for various ligands in the M-heads. Nucleotides and actin, when bound on these sites, or thiol reagents blocking the specific SH-groups on HC, change the HC state. The conformation of HC is also affected by LC located in the M-heads. LCs of the regulatory class participate in the physiological mechanisms which control the degree of interaction between M and A and this control is the molecular basis of the regulation of muscle contraction. In the first parts of this review studies showing the mode of action of LCs — the natural modifiers of the AM complex, and their function in the myosin-linked calcium regulation are discussed.

General views on molecular mechanisms of calcium control.

Classic schemes for the AM ATPase reaction include a cycle of several steps: ATP induced AM dissociation, ATP hydrolysis followed by a M to A reassociation and by a sequence of product release steps. In this sequence the affinity for nucleotides on the myosin ATPase site gradually decreases and that for actin on the actin binding site (A-site) on M correspondingly increases, resulting in a "rigor" AM complex formation (Lymn and Taylor 1971; Chock et al. 1976). This process is seemingly a result of a reciprocal allosteric interaction between the ATPase site and the A-site via some HC conformation changes. Various nucleotides as well as P_i bound at the ATPase site are known to induce such changes and to weaken the A-site affinity for A to a certain degree (Morales and Botts 1979).

A recent and more sophisticated scheme for AM ATPase (Stein et al. 1979) has been based on the observation that, at sufficiently high actin concentrations, a significant part of AS1 complex does not dissociate in the presence of ATP and S1 remains bound to actin throughout the complete ATPase cycle. Instead of ATP-induced dissociation, which is actually observed *in vitro*, a weak interaction between the A-sites and actin is assumed to take place during the initial steps of the reaction *in vivo* and under specific conditions *in vitro*. The affinity of M to A becomes strong at the P_i release step. The M to A association constants increase

stepwise in the sequence: $K_{a3} \sim K_{a13} \sim K_{a14} \ll K_{a15} < K_{a2}$ (in the authors' notation), where K_a characterizes the M—A affinities in the intermediates: K_{a3} in AM.ATP; K_{a13} in the AM complex unable to release products; K_{a14} in AM releasing P_i; K_{a15} in AM releasing ADP; K_{a2} in AM without any bound nucleotide and products. In steady state studies this model is experimentally indistinguishable from the "classic" ones (Marston and Taylor 1980). The double reciprocal plot of the inverse values of ATPase rate versus actin concentration gives a maximum turnover rate, V_m, and an empirical apparent association constant, K_a, (or $K_a^{-1} = K_m$ for actin) the latter depending on the affinities of various myosin intermediates for actin (K_a with an index).

The new description implies no fundamental revision of the principles on which the AM ATPase mechanism is based. Any changes in transition rates (an increase in k) between the myosin intermediates in the presence of actin are induced by interaction of the A-site with actin and they therefore depend on the affinity contants, K_a, for the corresponding M intermediates. The equilibrium constants for the transition steps: $K = k_+/k_-$ are entirely determined by the free energy drop between the states, i.e. by the ratios of the corresponding K_a's (Eisenberg and Greene 1980). The rate constats, k, also depend on both the spatial positions of M bridges and the rate of conformation changes, however, any change in K_a-s unavoidably induces corresponding changes in the rate constants (k).

These general considerations together with the more recent experimental data constitute the basis for some current ideas about the mechanisms of actin- and myosin-linked calcium regulation discussed in a recent review by Adelstein and Eisenberg (1980). According to these authors, the regulatory TN—TM system on actin (and hence Ca-ion bound to TN) can modulate K_a of all the M intermediates, but preferentially the strong K_{a15} of the state which releases ADP. Indeed, the K_a values for weakly interacting M states (K_{a3}, K_{a13}, K_{a14}) decrease insignificantly on Ca^{2+} removal (from 2.3×10^{-4} mol^{-1}.l to 1.3×10^{-4} mol^{-1}.l, whereas the AM ATPase rate decreases by 96%: it is the P_i release which is inhibited (Chalovich et al. 1981). It has also been shown that Ca^{2+} markedly enhances the interaction of the S1—ADP complex with regulated actin by increasing the relative amounts of strong-binding sites on actin (Greene and Eisenberg 1980). It may be suggested that both the inhibition of P_i release and the weakening of S1. ADP binding to actin by TN—TM (both reversed by Ca^{2+}) may have a common origin: an increase in the free energy of the AS1. ADP state (relative to AS1.ADP.P_i), i.e. a decrease in K_{a15}. The K value of the P_i release step must also decrease concomitantly, e.g. via k_+ inhibition, since K_{a15}/K_{a14} decreases. Thus there is no need to contrast changes in A-to-M affinities with changes in the product release rate in the calcium regulation: the former determine the latter. It has been assumed (Adelstein and Eisenberg 1980) that in the myosin-linked calcium regulation, direct Ca^{2+} binding to myosin or the calcium dependent myosin phosphorylation can also selectively

affect both the K_a value and the transition rates for different myosin states.

Certain chemical modifications of the skeletal myosin HC induce a sharp increase in the overall myosin affinity for actin. Heavy meromyosin (HMM), with one specific thiol group on its HC blocked, loses the ability to dissociate from the regulated actin in ATP-containing, calcium-deficient media; its actin-activated ATPase in these media is not decreased (Shibata-Sekiya and Tonomura 1975) and HMM particles sticking to thin filaments are seen at 90° in the electron microscope (Hayashi et al. 1973). This indicates a substantial increase in K_{a3}, K_{a13}, and K_{a14}, respectively, because under this angle the contact between A and M in ATP-containing solution is ascribed solely to the weakly interacting (with A) M states (Eisenberg and Greene 1980). Thus the HC state can directly affect calcium regulation in skeletal muscle actomyosin.

Function of the essential (alkali) light chains.

The effects of the "essential", alkali-separable ELC subunits of skeletal myosin (LC1 and LC3) on the A—M interaction are more difficult to detect. These effects have however been demonstrated on isolated preparations of S1 containing only one type of LC (LC1 or LC3). The K_m values obtained in 6 mmol.l^{-1} KCl for actin-activation of the ATPase activity of S1 with LC1 and S1 with LC3 were 10 and 50 μmol.l^{-1} respectively; also, different values of both the activity and V_m were obtained. The two LC types were interchangeable. Exchange of LC1 for LC3 (or vice versa) in the same S1 type resulted in higher or lower values of K_m, depending on the type of LC introduced, whereas the S1 ATPase activity remained unaffected (Weeds and Taylor 1975). The values of the latter for S1 from fast and slow skeletal muscle differ from that for cardiac muscle and depend on the HC type only, rather than on the LC (Wagner and Weeds 1977). When ionic strength is increased, approaching its physiological level, the K_m values for both the described above S1 preparations rise and the difference between them disappears (Wagner et al. 1979). Ionic strength has also a profound effect on the Mg^{2+}-stimulated A—HMM ATPase: in 0.10—0.15 mol.l^{-1} KCl the ATPase is no more activated by Mg^{2+} and its activity decreases to only 5% of its level in a KCl-deficient solution (Leadbeater and Perry 1963). Also the AM ATPase activity of AM suspensions markedly decreases with ionic strength, whereas contractile activity parameters such as tension and velocity of contraction in the muscle fibres and threads, do not (Crooks and Cook 1977). Therefore, it would be premature to insist on the physiological significance of experiments with ELC exchange; however the ELC ability to influence the affinity of the A-site to A is to be considered as proven. Alkali LC themselves are not included in the A-site structure (Holt and Lowey 1975) and they do not change the ATPase activity of M alone. Therefore, we must admit that these LC modify the HC state which determines the degree of lowering

the A-site affinity to A by nucleotide bound at the ATPase site.

A common function of both ELC types is to increase the stability of the HC, of both its ATPase and A-sites (Wagner and Giniger 1981).

Molluscan regulatory light chains.

The LC2 subunits of the skeletal muscle myosin belong to the class of regulatory LCs (RLCs). Two moles of RLC are contained in one mole of myosin from various muscle types, i.e. one RLC in every M head. As a rule, half of this amount (1 mole of RLC) can be reversibly released by a mild treatment of M with no damage to the M ATPase activity or with an only partial decrease in it. RLCs differ from the alkali LC in their molecular weight, they bind calcium (when included in M or, sometimes, in a free state), and some of them can be phosphorylated by specific kinases. The RLC function was demonstrated for the first time on molluscan myosin. When one mole RLC is removed (by treatment with 10 mmol.l^{-1} EDTA at 4 °C) from scallop myosin, the actin-activated ATPase rises in the presence of EDTA to levels similar to those observed in Ca^{2+}-containing media. Thus, the calcium sensitivity (Ca-sensitivity) of the AM ATPase becomes entirely lost. It is fully restored by reassociation of the removed RLC with myosin (free, in AM or in myofibrils). This desensitization to calcium is followed by a (reversible) loss of one calcium binding site on M. The actin-activated ATPase of HMM from desensitized scallop myosin (M_{sc}) is also calcium insensitive (Szent-Györgyi et al. 1973).

Other important experiments showed that RLCs from various muscles are able to replace one of the own RLC in M_{sc} and to restore (partially or fully) the Ca-sensitivity of the AM ATPase (Kendrick—Jones et al. 1976; Kendrick—Jones 1974). Thus, a common RLC property was demonstrated: when introduced in M_{sc} (i.e. in a system with a myosin Ca-control) various RLCs fulfil a function analogous to that of the TN—TM complex on actin; they change the A-site affinity to A depending on Ca^{2+} concentration. RLCs exclusively affect the A-site and no other sites or properties of the myosin molecule, as evidenced by desensitization of the soluble A—HMM ATPase. Different RLCs have equal affinities for desensitized M_{sc} in AM: when isolated and pooled they become bound to AM proportionally to their content in the mixture. However, only RLCs from molluscan muscle having a "direct" myosin Ca-regulation are fully able to replace its own RLC in purified M_{sc} and to function normally. Molluscan RLCs are able to restore the Ca-sensitivity and both Ca^{2+}-binding sites (Ca-sites) of pure M_{sc} completely even after both originally bound RLCs had been removed (by EDTA treatment at 25°—30 °C). RLCs of other myosin types associate with scallop AM or with myofibrils only; under identical conditions smooth muscle (chicken gizzard) RLCs restore specific Ca-sites (with a lower affinity however) as well as Ca-sensitivity, the restoration being only partial. The LC2 and cardiac RLC do not restore

Ca-sites at all and they are able to partially resensitize AM_{sc} ATPase with only one of its own RLC replaced, i.e. only in the case when a single Ca-site is preserved (Sellers et al. 1980).

Experiments on myosin Ca-regulation, particularly all data on RLC Ca-sites, have shown both similarity and specificities of diverse RLCs and, especially, of functional properties of HC. Seemingly all RLCs have one Ca-site per molecule. The affinity of this site for Ca^{2+} is increased ($K_d = 10^{-7}$—10^{-8} mol.l^{-1}) in RLC remaining in situ; Ca-sites are therefore easier to detect in myosin than in isolated RLCs. Ca-sites with a lower affinity ($K_d = 10^{-4}$—10^{-5} mol.l^{-1}) are found in isclated LC2 (Morimoto and Harrington 1974), in cardiac RLC (Kuwayama and Yagi 1977); they however are not detected in released molluscan RLC, probably because of their weak affinity. Using a spin-labelled isolated LC2 (Okamoto and Yagi 1976) it was shown that Ca-binding on the weak site induces a conformational transition in the subunit. This Ca-site also exists on LC2 in situ, here, its affinity increases to $K_d = 10^{-8}$ mol.l^{-1}, probably as a result of LC2—HC interaction. However, Ca-sites of this class on all RLC types (the molluscan RLC included) are unspecific and could hardly function as regulatory. They bind both Mg^{2+} and Mn^{2+} ions; under physiological conditions they must be saturated with Mg^{2+}. The rate of the Mg^{2+} replacement by Ca^{2+} is limited by the slow rate of Mg^{2+} dissociation and is several orders of magnitude lower than the velocity of muscle activation (Bagshaw and Reed 1977). Experimental data indicate that saturation of these Ca-sites with Ca^{2+} or Mg^{2+} is required for RLC and HC to associate. However, molluscan M-heads (and only these) possess supplementary Ca-sites of another class characterized by specificity and high affinity for Ca^{2+}. These sites do not bind Mg^{2+} or Mn^{2+} and serve evidently as calcium receptors in the myosin Ca-control. They appear upon binding of the molluscan RLC to M_{sc} HC but not to "foreign" HC of skeletal myosin. Apparently these Ca-sites are RLC associated; they however require the participation of certain HC residues in their structure or they become stabilized by HC (Bagshaw and Kendrick—Jones 1979). As confirmed by M_{sc} resensitization data, RLCs from smooth musle M seemingly also form such specific Ca-sites together with scallop HC. It is conceivable that a single foreign skeletal LC2 in M_{sc} functions in concert with the remaining "own" RLC which has a specific Ca-site itself (Chantler and Szent—Györgyi 1980).

The data available are insufficient to elucidate the precise regulatory mechanism based on Ca^{2+} binding to myosin. According to an early assumption (Szent—Györgyi et al. 1973), RLC without Ca^{2+} sterically blocks the A-site and opens it in the presence of Ca^{2+}. When RLC is missing, the A-site is also active in the absence of Ca^{2+}. The blocking mechanism has later on been conceived as some special steric arrangement of the two heads of the myosin molecule, hindering their interaction with actin in the absence of calcium. The insensitivity of the A—S1 scallop ATPase to calcium (in contrast to the calcium dependence of A—HMM

ATPase) seemed to confirm this concept. However, the AM—ATPase of single-headed M_{sc} was also found to be Ca-sensitive (Stafford et al. 1979). The difference between this and A—S1 ATPase can be explained as follows. Seemingly RLC can only function when fixed in an appropriate position by links to a certain HC region in the S1—S2 junction; this HC segment becomes destroyed in an isolated M-head. The S1—S2 junction has also been identified as the RLC binding region in cardiac skeletal muscle M (Kuwayama and Yagi 1980). Influence of RLC on the HC conformation in the S1—S2 junctional region (Bagshaw 1977) can account for the effect of a single RLC on the states of both heads in a M molecule.

Regulatory light chains in skeletal and smooth muscle myosins.

Experiments with the removal of LC2 from skeletal myosin contribute to our understanding of the RLC function. After a reversible extraction of one mole of LC2 from one mole of M by DTNB treatment both the AM ATPase activity and K_m^{-1} value decrease 3.6-fold without lowering the ATPase of M alone (Hozumi and Hotta 1978); the A-HMM ATPase decreases 5-fold (Werber and Oplatka 1974). Apparently the steric-blocking hypothesis for M_{sc} may hardly be applied to the funcion of LC2 in skeletal M.

However, it should be considered that changes in the HC state may not only be induced by the RLC absence itself but by the very process of their extraction as well. Exactly this was shown in studies on M_{sc}. On the removal of a single RLC from M_{sc} the AM ATPase activity (in the presence of Ca^{2+}) did not change (Szent—Györgyi et al. 1973) or dropped by 29% (Kendrick—Jones et al. 1976); after the release of both RLC however, it decreased 2 to 4-fold (Chantler and Szent—Györgyi 1980) as did the tension of glycerinated fibers (Suzuki et al. 1980). In tension measurements, the Ca-sensitivity decrease immediately follows the total RLC removal, and the tension drop develops with a 10 minutes lag, it therefore may be induced by a different HC conformation change. Removal of single LC2 from skeletal muscle M by a mild treatment (EDTA only, 35 °C) does not seem to affect the HC: the tension development by LC2-deficient AM threads is unimpaired (Srivastava et al. 1980). We infer that under the mild conditions of extraction of single RLC skeletal muscle myosin HC is as stable as the molluscan HC, and the abovementioned conformational change of the slow-developing kind does probably not occur.

Regardless of the noted ambiguities in the interpretation of the effects of LC2 release, a concept based on these effects emerges: the function of LC2 (and possible of other RLCs) is to adjust the HC and the A-site state to definite M to A affinity values, which, in the case of an actin-linked Ca-control, are determined by the affinity sites on actin. This possibility was tested in experiments on AM complex of regulated actin and LC2 deficient myosin at various A:M ratios and

Ca^{2+} concentrations (Pemrick 1977). At low A:M ratios the ATPase activity of LC2-deficient AM is reduced (depending on the conditions) by 20—60% and requires higher Ca^{2+} concentrations for its half-maximal activation. This decrease in the apparent affinity of TN in the thin filaments for Ca^{2+} seems to result from a decrease in the affinity of myosin intermediates to actin. At high A:M ratios (as in the studies on AM threads with A:M molar ratio of 4:1) the ATPase drop upon LC2 removal was substantially reduced. It has been concluded that "the LC2 stabilizes a particular conformation of native myosin which enhances actin activation" (Pemrick 1977).

This hypothesis appeared to be more substantiated when a different and more complicated myosin-linked Ca-regulation system in the smooth and skeletal muscle became discovered. RLCs in the smooth muscle enhance the AM ATPase following their phosphorylation and not in response to a direct Ca^{2+} binding. A specific RLC kinase transferring phosphoryl on RLC is Ca^{2+}-activated and a slow-working RLC phosphatase abolishes the activation of ATPase. We shall discuss some aspects of possible mode of action of phosphorylated RLC in the calcium regulation, referring to a few examples, without attempting to review all studies on the topics.

To understanding this mode of action data on phosphorylation of skeletal LC2 are important although their physiological significance still remains unclear. When 50% of the total LC2 amount in the skeletal muscle M becomes phosphorylated the AM ATPase activity (with regulated actin) is nearly doubled independent of the Ca^{2+} concentration and without any shift in the Ca^{2+} concentration required for half-maximal activity (Pemrick 1980). With 75% LC2 phosphorylated the K_m value for the AM ATPase is nearly fourfold reduced; V_m of the AM ATPase and the activity of Mg^{2+}-or K^+-activated M ATPase do not change. A similar decrease in K_m occurs for the A-HMM ATPase after LC2 phosphorylation in HMM. Phosphorylation seemingly enhances some of the A-site affinities. As stressed by the author, the effect is measurable at a low actin activation only, namely at actin concentrations (0.004 to 0.02 $mg.ml^{-1}$) below the K_m value for actin for unphosphorylated M (1.3 $\mu mol.l^{-1}$ actin). In a previous study by Morgan et al. (1976) no effect of LC2 phosphorylation on the skeletal AM-and A-HMM ATPase could be detected. The reason for this discrepancy may be a too high actin concentration (in the range of 3 to 10 $mg.ml^{-1}$) used, which presumably exceeded the concentration required for half-maximal activation of M ATPase (Pemrick 1980).

The generation of the isometric tension in ATP-activated AM threads in the absence of TN was calcium insensitive, i.e. devoid of any kind of the myosin-linked calcium regulation (Crooks and Cooke 1977). This is fairly consistent with the AM ATPase model mentioned above (Eisenberg and Greene 1980). Under isometric conditions many of the tension-developing myosin bridges oscillate between the weakly actin-associated states, and they cannot complete the ATPase cycle: they

dissociate from the thin filaments without splitting ATP and without releasing products. That is why isometric tension may be insensitive to the phosphorylation effect which can manifest itself as an A-site affinity increase at the product release steps (an increase in K_{a15}, K_{a2} and in the corresponding P_i and ADP release rates).

The existence of a regulation via LC2 phosphorylation in skeletal muscle is confirmed by direct recording of phosphate incorporation into LC2 upon activation of contraction and a decrease in its content during relaxation of frog muscle *in vivo* (Barany et al. 1979). In relation to the actin-associated regulation this system may fulfil an auxiliary function only, inducing supplementary ATPase and tension enhancement after the muscle has become activated.

The basic role of the RLC phosphorylation-associated calcium control system in smooth muscle is well known. Its essential features have been established in a number of papers and reviews (Sherry et al. 1978; Sobieszek 1977; Small and Sobieszek 1977; Sparrow et al. 1981; Barany and Barany 1980). Dephosphorylated chicken gizzard and pig stomach myosin and AM preparations purified from RLC kinase exhibit an ATPase which is low at any Ca^{2+} concentration. The AM activity increases in an exact proportion to the amount of phosphate incorporated into M and levels off at two moles of phosphate per mole myosin. The ATPase of phosphorylated AM is calcium independent, it decreases upon dephosphorylation of M (Sobieszek 1977).

The existence in smooth muscle of an actin associated Ca-regulation system (Ebashi 1980) is under question. However, both kinds of regulation can be observed simultaneously on artificial systems assembled *in vitro*. Superprecipitation experiments described by Ikebe et al. (1977) may serve as a good example of a regulatory "adaptation" of myosin to the actin state. A hybrid AM reconstituted from phosphorylated gizzard M and pure skeletal muscle A exhibits superprecipitation (with ATP) of a moderate rate and extent, independent of calcium. In the presense of TN—TM and EGTA the reaction is completely inhibited, being strongly enhanced with calcium: M is rightly adjusted by its phospho-RLC to the Ca-regulated actin state.

It can be concluded that RLC of smooth muscle myosin only impart the required affinity to the A-site when they are phosphorylated. Are these RLCs, like the molluscan ones, inhibitors of the AM ATPase and is their phosphorylation a derepression like the specific Ca^{2+} binding in molluscan myosin? To answer this question a removal of the RLC is required. The RLC of smooth and cardiac muscle myosins cannot be extracted by a mild treatment. Therefore, a proteolytic degradation of RLC has been used in these muscle types. The obtained results are contradictory. Upon treatment with papain a notorious increase in the ATPase of gizzard AM was observed even in the presence of calcium and kinase; for the gizzard HMM-A ATPase there was a threefold increase (Seidel 1978; Sobieszek and Small 1976). The ATPase of gizzard S1 obtained by papain treatment is

actin-activated (Marston and Tailor 1978). Also papain treatment markedly enhances the Mg^{2+}-M ATPase. Based on these results the view prevails that the smooth muscle RLCs inhibit AM ATPase which in turn is derepressed by RLC phosphorylation (Adelstein and Eisenberg 1980). We do not share this opinion. Papain alters the ATPase site during RLC destruction, enhancing the gizzard Mg^{2+}-ATPase of M alone nearly fourfold (Seidel 1978). Similarly a specific protease impairs the ATPase site of cardiac myosin and causes a conformational change which mimics the effect of modification of the "essential" SH—2 group on the HC; the Mg^{2+}-ATPase of M rises by 50—75% (Malhotra et al. 1979). As a rule, such a rise reflects an increase in K_m (for ATP) $= (k_{-1} + k_{+2})/k_{+1}$ where k_{+2} increases along with the rates of the limiting steps of the M ATPase cycle and with its turnover rates. Identical M states and transitions between them occur in the AM ATPase, and alteration of the ATPase site likely induces enhancement of both the limiting transition steps and AM ATPase turnover rate (V_m) as in the M ATPase. Moreover, with the increase in K_m for ATP the steady-state nucleotide concentration (the mean "occupation time") on the ATPase site decreases, and thus the inhibitory effect of nucleotide on the A-site affinities becomes weakened. Consequently, especially in a cardiac system with regulated actin (Malhotra et al. 1979) a supplementary activation by the "rigor" AM complexes may take place and the apparent K_m for actin decreases fivefold. All these changes are a result of an alteration of the ATPase site, rather than of RLC destruction. That is why conclusions drawn based on the use of papain or any other proteolytic enzymes affecting the ATPase site and enhancing the M ATPase, cannot be considered reliable (Seidel 1978).

On the other hand, after chymotrypsin or trypsin degradation of RLC the M ATPase is not enhanced; the actin activated ATPase of gizzard M or HMM markedly decreases in the presence of calcium and kinase (Seidel 1978) or drops to a low level similar to that found in dephosphorylated control AM (Sobieszek and Small 1976). Experiments in which the time course of trypsin degradation of gizzard RLC was carefully controlled (Okamoto and Sekine 1978) showed a net drop of the actin-activated ATPase of phosphorylated M in the presence of Ca^{2+} (with regulated skeletal actin) to a low level characteristic of undegraded AM in the presence of EGTA, coinciding with a complete RLC degradation and occurring before any alteration of the HC. These results characterize phosphorylated gizzard RLC as activators of the AM ATPase, whereas the unphosphorylated subunits have no effect on the A-site.

Hypothesis on myosin-linked regulation mechanism.

The above observation enables us to suggest an alternative to the hypothesis of steric blocking of the A-site. The weakness of the latter concept, which has been

especially adapted to the molluscan Ca-regulation, becomes evident if we realize that myosin already possesses an inherent mechanism to inhibit the A-site: allosteric suppression of the A-site affinity for actin by the bound nucleotide mentioned above. We suppose that RLCs are able to modulate the degree of this suppression by affecting the HC state. The strength of the RLC effect changes when they receive a "signal" consisting of specific Ca^{2+}-binding or RLC phosphorylation. The ability of LC2 to enhance the rates of conformational transitions induced in HC of skeletal M by ATP or ADP binding has already been shown (Garland and Cheung 1979). In molluscan HC deprived of RLC the effect exerted by nucleotide on the A-site is weak: both the affinity for actin (e.g., K_{a15} of the M ADP intermediate) and the AM ATPase are high. RLCs (own or foreign) enhance the effect of nucleotide; in their presence both the K_a value and the ATPase are low. Specific Ca^{2+} binding releases HCs from the inhibitory RLC impact and brings them closer to the RLC-deficient state. This would mean that Ca^{2+}-binding on the functional RLC site reduces the interaction between certain specific RLC and HC regions in the M-head. Cardiac HCs possess such a region close to the S1—S2 link; the state of this segment affects the A-site affinities, and a modification of the HC region may increase the K_m value for the AM ATPase four — or six-fold (Hiratsuka 1981). This specific RLC—HC affinity is thought to have no relation to the affinity of isolated RLC in solution for RLC-deficient M-heads which is equal for different types of RLCs capable or not to play a regulatory role in molluscan myosin. The latter interaction of a collision type seems to require Ca^{2+} binding to nonspecific sites (Bagshaw and Kendrick—Jones 1979).

Skeletal and smooth muscle HCs (unlike the molluscan ones) are good transmitters of nucleotide influence on the A-site and in the absence of RLC their AM ATPases and K_m^{-1} are markedly reduced (skeletal muscle HC) or almost totally inhibited (smooth muscle HC). Their RLCs (in smooth muscle when phosphorylated only) suppress the nucleotide effect on the A-site to a physiologically required degree and thus enhance the AM ATPase. As discussed before, the data for cardiac M (Malhotra et al. 1979) can be explained in terms of an alteration of the ATPase site itself under the experimental condition used.

Phosphorylation of RLC further enhances the AM ATPase by strengthening the specific RLC—HC interaction to a moderate degree (LC2), or in an "all or none" fashion (smooth muscle RLC). HCs of both the skeletal and smooth muscles are thus not adapted to a direct myosin-linked Ca-regulation. According to this scheme phosphorylation and specific Ca^{2+} binding would counteract each other in a case of their simultaneous action on the same RLC—HC interaction since they would change its degree in opposite directions. This does not seem to take ever place in natural systems.

The types of both RLC and HC determine which of the two signals is used for

RLC function. The RLCs of skeletal and smooth muscles may be phosphorylated, they however do not form specific Ca-sites with their own HC. Molluscan RLCs form such a site but they cannot be phosphorylated. However, in an artificial *in vitro* system phosphorylated gizzard RLCs can be inserted in the scallop M-head (RLC deficient) where these RLCs form specific Ca-sites, inhibit the AM ATPase in the absence of Ca^{2+} and confer the Ca-sensitivity to the HC. With dephosphorylated gizzard RLC the AM_{sc} ATPase is inhibited in EGTA media to 0.04; in calcium media, its activity increases to 0.80, but in calcium with phosphorylated gizzard RLC to only 0.67 (μmoles $P_i.mg^{-1}.min^{-1}$) (Sellers et al. 1980). RLC phosphorylation reduces the calcium effect.

The Ca-regulation system of vascular myosin represents a particular case (Chacko et al. 1977). With pure actin, phosphorylated M devoid of kinase exhibits a high AM ATPase with a net Ca-sensitivity, whereas dephosphorylated M gives a very low AM ATPase with only traces of Ca-sensitivity. Based on the above working hypothesis of ours one may suppose that this M contains an isoenzyme with HC and RLC of the molluscan type, which forms coaggregates during purification and then works cooperatively with the prevailing myosin species of a "gizzard" type.

To conclude our discussion on RLC functions it may be stated that the calcium regulation of the AM ATPase is a primary function of the RLC in most myosins. Both myosin-linked Ca-sensitive mechanisms involve two stages: 1. Ca-binding to RLCs, or their phosphorylation induce conformational changes in the RLCs (Okamoto and Yagi 1976). 2. These transitions result in changes in the A-site affinity for actin via a steric rearrangement or via an allosteric modulation of the HC by RLC. In systems in which the principal regulatory function is actin-associated, the myosin control mechanism adjusts the A-site affinity to the level required for the binding sites on actin.

Modification of the actomyosin ATPase via myosin rod.

It has recently been shown that the ATPase activity of skeletal muscle AM can be significantly altered by various factors that obviously have no direct effect on RLC or HC in the M-head region. It was e.g. demonstrated that the ATPase can be affected by a chemical modification of the myosin rod or by binding of certain proteins to this part of the M molecule.

In general reagents blocking SH-groups of the HC change their state, increasing the A-site affinity for actin. In works referred to above (Shibata—Sekiya and Tonomura 1975; Hayashi et al. 1973) a single SH-group belonging to one of the myosin HC was blocked by PCMB. As a result, the ability of the myosin molecule to dissociate from actin upon ATP binding was entirely lost. S1 derived from PCMB-treated myosin retains the properties of the latter; hence the reactive SH-group belongs to the M-head.

At the same time, in studies on the alkylation of myosin SH-groups by M-ethyl-maleimide (NEM), an essentially different kind of ATPase modification was found. Natural AM incubated with NEM at 25 °C lost Ca-sensitivity of its ATPase before any decrease in the activity could be observed. Similar results were obtained with reconstituted AM formed from NEM-treated myosin, although in this case the AM ATPase was lowered in parallel with the loss of Ca-sensitivity (Daniel and Hartshorne 1973). However, remarkably enough, S1 derived from natural AM treated in this way exhibited AS1 ATPase with an intact Ca-sensitivity. It may be supposed that the modified SH-groups responsible for the observed effect are located on the rod portion of M molecule which is absent in S1. This suggestion has been confirmed in the studies of Yamashita's group.

When natural AM is incubated under defined conditions (0 °C; 0.1 mmol.l^{-1} NEM, no KCl), its Mg^{2+}-ATPase increases in the initial period 6—10 times and somewhat more in the presence of calcium and EGTA, respectively; the Ca-sensitivity diminishes by a factor of 3, but does not disappear. These and some other data indicate that A—M affinity in the modified complex increases. A double reciprocal plot of the ATPase rate versus actin concentration for this complex gives an increased K_m^{-1} value (Yamashita et al. 1975; Yamashita and Horigome 1977; Horigome and Yamashita 1977). A single specific thiol group most reactive to NEM under the specific conditions used, termed "S_a"-group, is responsible for this effect. The S_a-group has been localized in the M rod hinge, close to the S2 segment of the HMM rod part (Horigome and Yamashita 1979a). The authors assume that alkylation of the S_a-group results in a change in the conformation of the rod hinge segment; the M-head (belonging to an adjacent M molecule in the thick filament) sticking to this segment can swing away from it. As a result, the local concentration of M heads near the thin filaments increases (Horigome and Yamashita 1979b).

This explanation deserves some comments. Contacts between the M head and the thick filament surface have in fact been demonstrated (Sutoh and Harrington 1977); however these contacts by no way interfere with the A—M interaction. Any restrain of this interaction, if occurring in intact thick filaments (native or reconstituted), should be observed as a decrease in AM ATPase in relation to the ATPase activities of soluble A—HMM and A—S1 devoid of the "binding" M rods. Experimental data fail to show such divergences. The turnover durations (at 25°) for the Mg^{2+}ATPase of rabbit muscle A—HMM, AM and myofibrils (at comparable actin concentrations) are 1.2—1.4s (Inoue et al. 1973), 0.7 s (Yamashita et al. 1975) and 0.5 s (Schaub and Watterson 1973), respectively. For the ATPase of A—S1 and myofibrils from chicken white muscle values of 0.05 and 0.08, have been reported (Marston and Taylor 1980), respectively. Regardless of the experimental error, it is evident that the differences are by no means comparable to the six- or ten-fold activity increase in the AM ATPase after NEM treatment. Moreover, it has been shown in a recent study (Reisler 1980) that the actin-activa-

ted Mg^{2+}-ATPase of myosin in a minifilamentous form and that of HMM are kinetically equivalent (having identical values of both V_m and K_m). For that reason an "active" binding of the M-rod in the hinge region may rather be proposed, by which the HMM-part of the molecule becomes protruded towards the thin filaments. The following experiment of the authors is consistent with such a mechanism. Natural AM in agarose gel, where the protein aggregates are densely packed on pore surface, and no chemical or conformational changes in protein seem to occur, exhibited an enhanced ATPase activity exactly identical in its magnitude at various Mg^{2+} concentrations to the activity of NEM-treated AM in the same media. This effect may be due to externally induced approaching of thick to thin filaments in compressed aggregates.

Certain results of studies of the NEM effects cannot be explained solely in terms of the increase in the effective concentration of M heads. For example, a Ca-sensitivity drop, even if moderate, is a generally recognized manifestation of a rise in the A-site affinity. Thus the enhancement of the ATPase rate of NEM-treated AM seems to be caused by two independent factors: the already mentioned rise of the local M-heads concentration and an increase in the affinity of myosin for actin. The latter can be regarded as a result of an active outward movement of M-heads assuming a certain still unexplored myosin property. This property is expressed in the phenomenon of a direct mechanical action on the ATPase of contractile systems. Seemingly, an external force (as also the active contractile force itself), which promotes and accelerates the outward movement (tilt) of myosin bridges augments conformational transitions coupled to these movements and increases the ATPase rate; conversely, a force applied in the direction opposite to the movement of the bridges inhibits the AM ATPase. It has been observed that HMM solution streaming along an immobilized actin sheet in the direction of possible M-head tilt on actin enhances the A—HMM ATPase (Yano and Shimizu 1978). A rapid stretch of a glycerinated muscle fibre in ATP media can almost stop the ATPase cycles which are renewed upon a quick release of the fibre (Arata et al. 1978). These effects are described as a strong dependence of the rates of conformational transitions in the M heads on geometric positions of bridges in relation to the interaction sites on actin. There is no generally accepted theory of these phenomena. Elastic crossbridge models are based on an assumption that the contractile force produces an elastic deformation of the M head: the free energy is transmitted to the head structure and is used mechanically (Eisenberg and Hill 1978). Consequently, an external force applied to the bridges can do the same. Using an oversimplification it may be said that the contractile system responds to the force as if the energy introduced in the head structure were to contribute to lower the reaction barrier (and to enhance the reaction rate constants k) when the reaction-associated head movement is accelerated, or as if it were "utilized" to rise the barrier and to inhibit the reaction when the movement is hindered.

Recent kinetic data (Stein et al. 1979) have shown that, during the ATPase cycle, AM never becomes completely dissociated: the A—M binding becomes stronger after each successive product release step of a single cycle. Let us speculate that the actively protruding M heads are pressed against the thin filaments by a force applied transversally to the filament axis, and that this force induces an elastic deformation of the heads. Then the force would contribute to strengthen the A—M binding similarly. As in the bridge movements, it would enhance the equilibrium and rate constants (K_{15}, K_{a14}, and the corresponding K and k, see part 2 of the present review) of all those reaction steps at which M and A associate, or their mutual affinity increases. If this were right, a mechanically induced K_m^{-1} increase would be possible.

Another type of modification of the myosin rod part is its specific interaction with some proteins, first of all with paramyosin (PM). PM is a rod-like, highly helix-coiled protein (220,000 daltons, 135 nm in length) found in some insect and molluscan muscles. PM forms the core of the thick filaments in the molluscan catch muscles; this core is covered by a layer of myosin molecules. PM of the Clam adductor muscle, when coprecipitated with myosin (skeletal or molluscan), inhibits the AM ATPase of the M complex with pure actin. At saturating PM concentrations the ATPase activity decreases by 60—75%. From 1/v, vs 1/[A] plot it appears that PM is a competitive inhibitor of actin-activation. It decreases the K_m^{-1} value, whereas V_m remains unchanged. However, PM is not bound to the A-site, since it does not affect the A—HMM and A—S1 ATPase. The effect of PM is specific: it only occurs following a rapid coprecipitation resulting in the appearance of cofilaments with a smooth surface and no protruding M heads. No effect is produced when the precipitation is slow or carried out in two separate steps for the two proteins, resulting in cofilaments with projections (M heads) on their surface. LMM added to the protein mixture diminishes the effect. PM molecules shortened by partial proteolytic degradation have an appreciably smaller effect (Epstein et al. 1976).

All these data taken together indicate that PM is bound to M rod (the lack of the effect on both A—HMM and A—S1), and that both the LMM part of the rod (the effect of LMM) and its S2 segment (the lack of M heads on cofilament surface) are involved in this binding. According to the authors the PM binding to the LMM part of the rod is strong and stable; in addition, there is a specific site of a weaker equilibrium interaction with PM on the S2 segment. On the latter site PM "competes" with actin "for determination of the state" of myosin.

It is supposed that neither the A-site affinity nor the HC conformation are affected by PM. Rather, PM changes the configuration of the M molecules in the filament: being attached to both S2 and LMM rod segments (on both sides of the rod hinge) PM can reversibly shift a dissociated bridge away from the thin filements toward the surface of the thick filament. If the rates of both PM binding to and

dissociation from the S2 site are low enough, than at any given moment the fraction of the bridges with bound PM is excluded from the ATPase cycles. This corresponds to a lowering of the local concentration of M heads in the vicinity of thin filaments and, consequently, to a lowering of the M to A reassociation rate. Increasing the actin concentration counteracts the PM effect, since it diminishes the percentage of the dissociated bridges able to be declined from thin filaments. Thus the PM effect in supposedly solely based on M rod configuration changes (Epstein et al. 1975).

There are two other examples of myosin interaction with another protein that affects the AM ATPase. An inhibitory ("I") protein (50,000 daltons) from skeletal muscle reduces the ATPase of reconstituted AM with pure actin by 40—60%. It also decreases the A—HMM ATPase, but to a substantially lower degree. I-protein has been localized in the A-band of the sarcomere with the exception of its central zone; it is not bound to F-actin (Ohashi et al. 1977a, 1977b). Its effect reaches maximum level at approximately one mole I-protein bound to two moles myosin. This implies that I-protein is bound to the M rod, rather than to the M heads. In the presence of TN—TM the effect of I-protein disappears in calcium containing media, and persists in calcium deficient ones (Maruyama et al. 1977). The I-protein seems to affect the ATPase only when pure actin monomers are operating independently and interacting mainly with single M heads belonging to different molecules, or when (in the presence of TN—TM and without Ca^{2+}) A interacts with a few sparsely spaced bridges. Thus I-protein while retracting the bridges is unable to overcome the cooperativity of the actin binding to both heads of a myosin molecule or to neighbouring bridges. Its effect in an assembled contractile system increases the Ca-sensitivity in systems with actin-associated Ca-regulation.

Another inhibitory protein (LMM—R) is contained in routine LMM preparations. It has nothing common with I-protein. LMM—R is a protein *complex* (55,000 daltons). It includes a myosin-linked choline esterase and is presumably bound to the myosin rod in the hinge region. It is detached from the HC fragment after degradation of the hinge. In the presence of LMM—R the natural AM and myofibrillar ATPase decrease by 20—30% in calcium media; the K_m^{-1} value of the HMM—A ATPase is also slightly reduced. On the other hand, LMM—R induces a complete and reversible relaxation of glycerinated muscle fibres in contracting ATP media. Choline esterase inhibitors have an inverse and a much stronger effect on both the fibres and ATPase. They considerably reduce (by 50%) the Ca-sensivity of the myofibrillar ATPase and almost totally abolish the ability of the fibres to relax in calcium deficient ATP media. The ATPase of reconstituted AM is unaffected by these reagents. The contractility of the fibres is enhanced by acetylcholine (Ach). It has been suggested that a triggering protein system associated with the myosin rod and sensitive to Ach and to its analogues is able to enhance or reduce the A—M interaction in the contractile apparatus. This system

may include LMM—R and an Ach sensitive protein (Kalamkarova et al. 1975a; 1975b; Kalamkarova et al. 1982).

Following assumption concerning force generation involving an active movement of the bridges towards the thin filaments has been made (Barany et al. 1979). The movement may be caused by an electrostatic repulsion between the negatively charged thick filament and a similar charge, increased by RLC phosphorylation, on the M-heads.

The principal myosin regulatory mechanisms discussed in the first parts of our review have been based on a common property of proteins — their ability to undergo conformational transitions accompanied by affinity changes. On the contrary, the few effects observed *in vitro* and associated with the myosin rod are strictly specific for myosin. Their physiological role has not yet been elucidated. These effects may be related to specialized functions such as paramyosin participation in the catch muscle mechanism, the enhancement of the actin-associated Ca-sensitivity in skeletal muscle by a minor protein of the thick filaments, or possible direct involvement of acetyl-choline in the contractile process. One can suppose that in all these cases the modification of the AM ATPase is more gradual and entirely different in its nature from the ATPase control via RLC. In this mechanism of actin the ability of the myosin molecule to change its configuration may be utilized. All the rod effects observed so far and discussed herein concern solely skeletal muscle protein systems of the vertebrates.

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Abbreviations

A	— actin
A—HMM	— acto-heavy meromyosin
AM	— actomyosin
AS1 or S1A	— the first acto-subfragment of heavy meromyosin
A-site	— actin-binding site of myosin
[A]	— concentration of actin
Ca-sensitivity	— sensitivity of the AM ATPase to calcium ion
EGTA	— ethylene glycol bis (β-aminoethyl ether) — N,N,N′,N′, — tetraacetic acid
EDTA	— ethylene diamine tetraacetic acid
ELC	— essential light chains of myosin
HC	— heavy chains of myosin
HMM	— heavy meromyosin
K	— equilibrium constant of a reaction step
k	— rate constant
K_a	— association constant
K_m	— apparent dissociation constant of myosin actin in the presence of ATP (equal to the actin concentration at a half-maximal myosin ATPase activation)
K_m^{-1}	— apparent association constant of myosin actin in the presence of ATP
K_m for ATP	— Michaelis constant for ATP
K_d	— dissociation constant
LC	— light chains of myosin
LC1, LC3	— essential light chains of vertebrate skeletal myosin
LC2	— regulatory light chains of vertebrate skeletal myosin
LMM	— light meromyosin
M	— myosin
NEM	— N-ethylmaleimide
PCMB	— para-chloromercuribenzoate
RLC	— regulatory light chains
S1	— the first subfragment of heavy meromyosin
S2	— the second subfragment of heavy meromyosin
TN	— troponin
TN—TM	— troponin-tropomyosin complex
V_m	— maximal rate of the actomyosin ATPase
v	— rate of the ATPase