Cooperative Role of Two Sulfhydryl Groups in Myosin Adenosine Triphosphatase†

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ABSTRACT: Evidence is presented to show that inhibition of myosin MgATPase results from a specific interaction between the metal substrate and the two sulfhydryl sites SH_1 and SH_2 to form a myosin MgATP cyclic ternary complex. In the absence of nucleotide the SH_1 group reacts readily with *N*-ethylmaleimide while the SH_2 is buried and unreactive. Binding of free nucleotide (no divalent metal) exposes the SH_2 group but reaction at low excess of *N*-ethylmaleimide is still restricted to the SH_1 because of its higher reactivity. Under similar conditions binding of MgATP or MgADP protects the SH_1 site from the reaction, the former affording a high degree of protection to the SH_2 site as well. This protection was not observed with $MgCl_2$, MgAMP, CaADP, or ADP, indicating the specific nature of the interaction between Mg nucleotide and the sulfhydryl sites of myosin. Myosin MgATPase inhibition can be abolished by blocking either sulfhydryl group. It is proposed that actin activation of unmodified myosin results from the disruption of the cyclic inhibiting structure by binding at or in the vicinity of the SH_1 site. It is speculated that the hydrolysis of ATP lowers the stability of the myosin MgATP inhibiting complex, thereby allowing actin to interact with myosin to disrupt the myosin·MgADP·P_i cyclic complex. The possibility that the burst phenomenon is related to the chelate formed between the bivalent metal and the terminal and penultimate phosphoryl groups is also discussed.

Perhaps the most frequently reported property of the hydrolysis of ATP by myosin is its strong dependence on the nature of the cation present in the system. It is known, for example, that in the absence of Mg^{2+} (presence of EDTA) the ATPase activity of myosin is activated by K^+ and inhibited by Na^+ ions (Warren *et al.*, 1966; Seidel, 1969a). In the millimolar range, Ca^{2+} is found to activate the hydrolytic reaction whereas Mg^{2+} strongly inhibits the same reaction (Sugden and Nihei, 1969, and references therein). *In vitro*, under conditions similar to the physiological state of muscle (low ionic strength, 10^{-3} M MgATP) the ATPase activity of myosin is strongly inhibited, but if actin is added to the system this inhibition is abolished and the rate of hydrolytic cleavage is dramatically enhanced.

It is now known that the binding of ATP to myosin produces small time-dependent conformational changes in the globular "head" region of the molecule which have been detected by ultraviolet (uv) difference absorption spectroscopy (Yoshino *et al.*, 1972), fluorescence (Werber *et al.*, 1972), and spin-labeling (Seidel and Gergely, 1971). With these techniques the effects produced by binding either ATP or ADP to myosin are similar except that the perturbation with the former is initially greater. These small changes in conformation, however, apparently do not involve a large degree of unfolding since no changes in optical rotatory dispersion (ORD) or hydrodynamic properties of myosin have been detected in the presence of these ligands (Gratzer and Lowey, 1969; Godfrey and Harrington, 1970).

From chemical modification studies it is known that there are two particularly reactive cysteine residues per subunit generally referred to as the SH_1 and SH_2 groups, which are located at or near the active site of myosin (Sekine and Kielley, 1964; Sekine *et al.*, 1962; Yamaguchi and Sekine, 1966; Bailin and Bárány, 1972a). Blocking of the SH_1 sulfhydryl results in an activation of the CaATPase activity with a concomitant loss in the EDTA (K^+) ATPase activity (Sekine and Kielley, 1964). Blocking of both sulfhydryls, SH_1 and SH_2, eliminates both ATPase activities (Yamaguchi and Sekine, 1966). Seidel (1969b) has found that myosin preferentially blocked at the SH_1 or SH_2 exhibits a high level of CaATPase activity but no EDTA (K^+) activity, indicating that both sites are required for the latter activity, whereas either site alone is sufficient for the expression of CaATPase activity. Our recent results support these conclusions and have also demonstrated the requirement of both sulfhydryls for the inhibition of myosin ATPase in the presence of millimolar levels of MgATP (Burke *et al.*, 1973). It was suggested in that work that maximum inhibition and maximum activation of the rate of MgATP hydrolysis *in vivo* occurs in the millimolar range of Mg^{2+} through formation and disruption of a cyclic MgATP ternary complex with myosin which involves coordination with both the SH_1 and SH_2 sites.

The results to be presented below provide additional support for the above mechanism. From a study of the influence of various ligands on the reaction between NEM[1] and myosin, it is concluded that the formation of the inhibitory cyclic structure requires the presence of MgATP and the obligatory participation of both the SH_1 and SH_2 sites. We believe further that actin activates the myosin ATPase activity by binding in the vicinity of the SH_1 site thereby causing disruption of the inhibiting cyclic structure. Rapid dissociation of the myosin·MgADP·P_i complex then occurs as the result of the lowered binding affinity of MgADP·P_i to the SH_2 site.

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[1] Abbreviations used are: FDNB, fluorodinitrobenzene; DNP, dinitrophenyl; NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithobis(2-nitrobenzoate); HMM, heavy meromyosin; AMP-PNP, β,γ-imino-ATP; AMP-CPP, α,β-methylene-ATP.

Materials and Methods

Glass-distilled water was used throughout and inorganic salts and reagents were of analytical grade. FDNB and NEM were the products of Eastman Kodak Co. (Rochester, N. Y.) and ATP of the highest commercially available purity was purchased from Sigma Chemical Co. (St. Louis, Mo.). The preparation of myosin and actin has been described elsewhere (Godfrey and Harrington, 1970; Bailin and Bárány, 1972b).

Modification of SH_1 groups of myosin with FDNB was accomplished as follows: myosin (0.2% in 0.5 M KCl–0.01 M Tris–0.001 M EDTA (pH 8.3)) was treated with a fourfold molar excess of FDNB for 30–35 min at 5°. The reaction was terminated by precipitation of the protein with 15 volumes of cold water. The protein was then dissolved in 0.5 M KCl (pH 7.5) and precipitated twice with cold water. Reduction of the dinitrophenylated myosin (0.4%) was accomplished with a 100-fold molar excess of sodium dithionite at room temperature, pH 8.3, for 5–10 min. The reaction was terminated by precipitation of the protein with 15 volumes of cold water. The precipitated protein was collected by centrifugation and redissolved in 0.5 M KCl–0.001 M Tris (pH 7.8); the precipitation and resolubilization steps were repeated twice.

In order to obtain myosin preferentially blocked at the SH_2 site, dinitrophenylated myosin (1% in 0.5 M KCl–0.05 M Tris–0.001 M MgADP (pH 7.9)) was treated with a fourfold molar excess of NEM for 30 min at 5°. The reaction was terminated by precipitation as described above. The dinitrophenyl group was subsequently removed by treating the protein for 45–60 min with 0.05 M β-mercaptoethanol in 0.5 M KCl–0.01 M Tris–0.001 M EDTA (pH 8.5) under nitrogen and at room temperature (*cf.* Bailin and Bárány, 1972a; Shaltiel, 1967). The protein was subsequently precipitated with 15 volumes of cold water, centrifuged, and resolubilized in 0.5 M KCl–0.05 M Tris-His (pH 7.0) and dialyzed extensively against this solvent.

The reaction of myosin with NEM was accomplished under conditions similar to those reported by Sekine and Kielley (1964). Myosin (1%) in 0.5 M KCl–0.05 M Tris-His (pH 7.0) or 0.5 M KCl–0.05 M Tris (pH 7.9) was treated at 5° with either a four- or eightfold molar excess of NEM for the desired time. The reaction was terminated by a 20-fold dilution of the protein in 0.5 M KCl–10^{-3} M Tris (pH 7.5) containing at least a 50-fold excess of β-mercaptoethanol. The effect of ligands on the course of the reaction of myosin with NEM was studied under the same conditions. The concentration of the added ligand in the reaction mixture was 10^{-3} M.

We have observed that the reaction of NEM with myosin is less specific at 25° than at 0–5°. Apparently the relative rate of modification by NEM of the essential with respect to the nonessential sulfhydryl groups decreases with increasing temperature.

The Ca and EDTA ATPase activities of myosin were measured at 37° employing the procedures of Kielley and Bradley (1956) and Kielley *et al.* (1956). Actin activation of the ATPase of myosin and the MgATPase of myosin were measured in the pH-Stat at pH 7.9 and 25°.

Results

ATPase Activities of Modified Myosins. The reaction of FDNB with myosin produces in qualitative terms the same changes in its ATPase behavior as the reaction with NEM. Though perhaps less specific for the reactive sulfhydryl groups than NEM, the reaction with FDNB offers an important advantage of being easily reversed with subsequent full restoration of the Ca and EDTA ATPase activities to values characteristic of native myosin. This restoration of activity is achieved upon thiolysis (*e.g.*, treatment with β-mercaptoethanol) of the DNP group attached to the SH_1 site (Bailin and Bárány, 1972a). Furthermore, since the dinitrophenyl group is uncharged, we hoped that no significant alteration in the conformation of myosin would result, as apparently occurs with DTNB (Gazith *et al.*, 1970), which has also been used to reversibly block the SH_1 site (Seidel, 1969b).

The ATPase activities of myosin treated with a fourfold molar excess of FDNB are given in row 2 of Table I. As in the reaction with NEM (Sekine and Kielley, 1964), the Ca-ATPase activity is increased while the K^+ (EDTA) ATPase activity is significantly reduced (see also Bailin and Bárány, 1972a). These changes closely parallel those found by Bailin and Bárány (1972a) for myosin which had been stoichiometrically modified at its SH_1 by FDNB as shown from peptide analyses. Although FDNB is known to react with a number of amino acid residues, it has been shown that under mild reaction conditions FDNB exhibits a high degree of selectivity for specific sites in proteins. (For example, Shaltiel and Soria, 1969, have shown that it is possible to restrict the reaction of

TABLE I: Relative ATPase Activities of Modified Myosins.[a]

		EDTA (%)	Ca (%)
1	Control	100	100
2	SH_1-DNP / SH_2	21 ± 4	450 ± 70
3	SH_1 / (after thiolysis) / SH_2	77 ± 7	107 ± 5
4	SH_1-DNP (reduced) / SH_2	79 ± 5	360 ± 30
5	SH_1-DNP (reduced) + mercaptoethanol[b] / SH_2	79 ± 5	321 ± 10
6	SH_1-DNP / SH_2-NEM	0	84 ± 4
7	SH_1-NEM / SH_2-NEM	0	10
8	SH_1-DNP (reduced) / SH_2-NEM	5	88
9	SH_1 / SH_2-NEM	8	117 ± 30
10	SH_1-NEM / SH_2	10	250 ± 20

[a] Activity measurements were carried out at 37° (Kielley and Bradley, 1956; Kielley *et al.*, 1956). 100% CaATPase refers to 1.0 μmol of P_i mg^{-1} min^{-1} in 0.05 M KCl (pH 7.6). 100% EDTA ATPase refers to 3 μmol of P_i mg^{-1} min^{-1} in 0.8 M KCl (pH 7.6). [b] The SH_1-phenylenediamine derivative is stable to thiolysis conditions. No effect of reducing agent on control was observed.

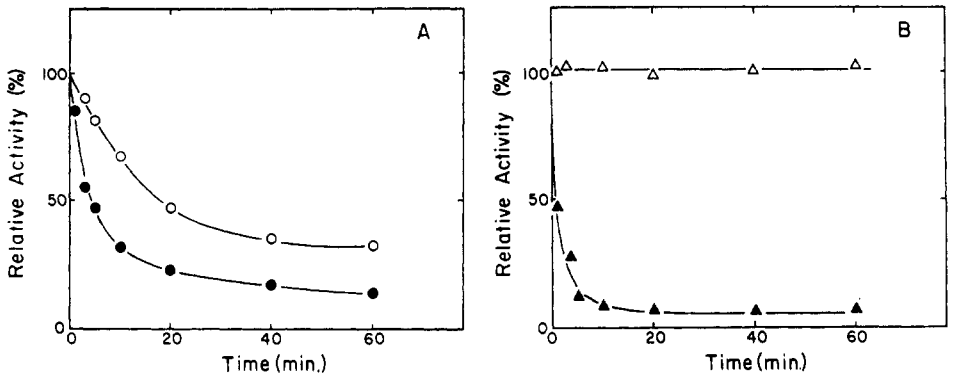

FIGURE 1: Relative ATPase activity of modified myosins reacted at pH 7.0 with an eightfold molar excess of NEM plotted as a function of time of modification: (A) SH_2-NEM myosin (recovered from SH_1-DNP, SH_2-NEM myosin) reacted with NEM in absence (○) and in presence of 10^{-3} M MgADP (●); (B) SH_1-NEM myosin reacted in absence (△) and in presence (▲) of MgADP.

FDNB with glyceraldehyde-3-phosphate dehydrogenase to one specific sulfhydryl group.) In our own work, we have observed no further change in CaATPase activity if SH_1-NEM blocked myosin is treated with a fourfold molar excess of FDNB or if SH_1-DNP myosin is treated with a fourfold molar excess of NEM, indicating that both FDNB and NEM are reacting with SH_1.

We have utilized the selective labeling of the SH_1 group with FDNB to subsequently modify the SH_2 group with NEM. Yamaguchi and Sekine (1966) have shown that SH_2 in SH_1-NEM myosin is apparently unavailable for reaction with NEM in the absence of ADP or its Mg complex. In the presence of MgATP or MgADP, SH_1-NEM myosin rapidly reacts at its SH_2 site with NEM, resulting in a marked reduction of the CaATPase activity (Yamaguchi and Sekine, 1966). We have found that SH_1-DNP myosin reacted under the same conditions with NEM also displays a large decrease in the CaATPase activity, indicating blocking of the SH_2 group to form SH_1-DNP, SH_2-NEM myosin. Removal of the DNP group by exposure to excess β-mercaptoethanol at pH 8.5 results in the formation of myosin selectively blocked at the SH_2 site. It may be noted that the CaATPase of SH_1-DNP, SH_2-NEM myosin (Table I, row 6) is significantly higher than that of SH_1-NEM, SH_2-NEM myosin (row 7). This discrepancy is most probably due to a lower efficiency of modification by NEM of SH_2 groups on SH_1-DNP than on SH_1-NEM myosin. For example, if 18% of SH_1-DNP myosin were not labeled on SH_2 this would account for the higher value of CaATPase. More extensive modification of SH_2 groups is easy to achieve either by higher ratios of NEM over myosin or prolonged modification time. Unfortunately on such extensive modification full recovery of ATPase activity following thiolysis of the DNP label is not possible. Thus, the optimum conditions for blocking of SH_2 on SH_1-DNP myosin were determined by the requirements of the thiolysis reaction, and precluded maximum depression of CaATPase activity.

The results in rows 6 and 9 of Table I necessitate careful analysis of the product of the thiolysis reaction and verification that the reaction truly yields SH_2-NEM myosin. Removal of the DNP label from SH_1 can be conveniently followed spectrophotometrically by monitoring the disappearance of an absorption band (at 340 mμ) characteristic for DNP derivatives (Shaltiel, 1967). After completion of thiolysis the isolated product of the reaction was again treated with NEM. Under conditions which allow preferential blocking of SH_1 (pH 7.0, no ADP) the reaction proceeded rather quickly leading to a rapid loss of CaATPase activity (Figure 1A). The time-dependent profile of this reaction was not dramatically affected by the presence of MgADP. Following Seidel (1969b), we interpret such reaction profiles to signify that SH_2 groups are indeed blocked and SH_1 free to react. In the alternative case, with SH_1 blocked and SH_2 free to react, no change in CaATPase is observed unless MgADP is added to the reaction system (Figure 1B; see also Yamaguchi and Sekine, 1966).

Two additional tests to differentiate between SH_1-NEM and SH_2-NEM myosin are provided by the markedly different dependencies of the CaATPase activities on temperature and ionic strength. Thus, the CaATPase activities of SH_1-NEM and SH_2-NEM myosin increase 42- and 18-fold, respectively (in 0.075 M KCl), over the temperature range 10–37°. At 25° their activities increase 5.5- and 2.6-fold, respectively, over a KCl concentration range from 0.075 to 1 M.

The above tests, remodification by NEM, and temperature and ionic strength dependencies of CaATPase allow for a clear distinction between the three possible systems: SH_1-NEM myosin, SH_2-NEM myosin, and a mixture composed primarily of SH_1-NEM, SH_2-NEM (that is, an inactivated protein), together with a residual fraction of SH_1-NEM myosin.

A comparison of SH_1-DNP myosin and SH_2-NEM shows distinct differences in their enzymic responses to CaATPase (Table I, rows 2 and 9) and to Mg^{2+} activation (Burke *et al.*, 1973). In agreement with the work of Seidel (1969b), our results indicate that both SH_1 and SH_2 are required for the expression of EDTA ATPase activity, whereas either site alone is sufficient for the maintenace of the CaATPase activity. Unlike Seidel (1969b), however, we find that the CaATPase activity of the SH_2-NEM modified myosin is equal to that of native myosin (Table I, row 9). The fact that under similar assay conditions (low ionic strength) Seidel (1969b) obtained lower values for CaATPase of the SH_2-NEM myosin may be due to a partial loss of activity following the DTNB treatment and the associated change in the conformation of myosin (Gazith *et al.*, 1970).

Measurement of the Ca and EDTA ATPase activities offered a convenient and unambiguous test for the type of sulfhydryl modified myosin produced in ligand mediated modification reactions. Thus, a decrease in EDTA ATPase reflects the extent of modification of either SH_1 or SH_2, while the difference between the levels of CaATPase activities of SH_1-NEM and SH_2-NEM myosin, shown in Table I (rows 9 and 10), identifies which of the two groups is involved in any specific reaction. The ionic strength and temperature

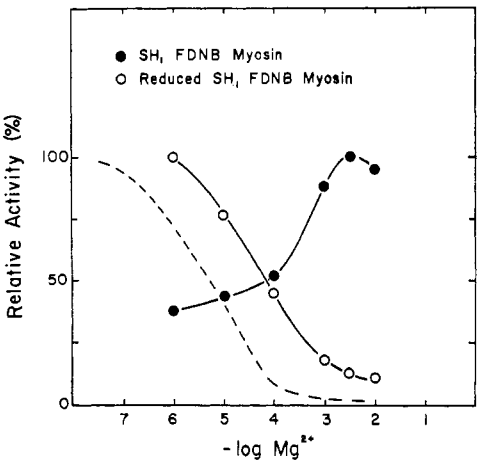

FIGURE 2: Relative ATPase activity of SH_1-DNP modified myosin and reduced SH_1-DNP modified myosin as a function of the concentration of added Mg^{2+}. The dashed line shows the behavior of native myosin. Activities were measured at 25° in 0.075 M KCl–1 mM ATP, in a pH-Stat at pH 7.9. 100% ATPase activity of SH_1-DNP myosin corresponds to 0.06 μmol of P_i mg^{-1} min^{-1} and that of reduced SH_1-DNP myosin to 0.12 μmol of P_i mg^{-1} min^{-1}.

dependencies of CaATPase activity and the remodification by NEM were used to verify these conclusions.

Our previous results (Burke *et al.*, 1973) on the effect of added Mg^{2+} on the ATPase activities of SH_1-NEM and SH_2-NEM blocked myosin demonstrated that both SH_1 and SH_2 groups are required to obtain the Mg^{2+} induced inhibition of ATPase activity. We have attributed this requirement of both sulfhydryl sites to a specific interaction between them and the bound MgATP substrate to form a cyclic ternary structure. This inhibiting structure, however, will not form if either sulfhydryl is modified.

Strong support for the presence of coordination between MgATP and the sulfhydryl site SH_1 is obtained by reducing the dinitrophenyl group of SH_1-DNP myosin to a phenylenediamine derivative. Since the phenylenediamine group should be an effective nucleophile capable of forming coordination structures, it was thought that this modification might restore the ATPase behavior to that of unmodified myosin. This expectation was borne out in the case of MgATPase activity (Figure 2) which is restored almost completely. Thus, apparently, the presence of a strong nucleophile at the SH_1 site restores the capacity of this site to form the inhibiting cyclic structure with MgATP. The fact that the CaATPase activity remains highly activated (Table I, row 4) indicates that the SH_1 site remains blocked and the binding interaction of Ca-ATP with myosin is altered by this modification.

Modification of Myosin with NEM in the Presence of Ligands. If inhibition of the ATPase activity of myosin at high MgATP concentrations (10^{-3} M) is due to a specific interaction between MgATP and the two sulfhydryl sites, SH_1 and SH_2, we would expect the modification of these sites to be specifically affected by the presence of MgATP or MgADP. The effect of the latter, however, should be smaller because of its weaker binding affinity for myosin (Schliselfeld and Bárány, 1968; Malik *et al.*, 1972). NEM is the reagent of choice since its specificity for SH_1 and SH_2 sites of myosin at high ionic strength has been well documented (Sekine and Kielley, 1964; Sekine *et al.*, 1962; Yamaguchi and Sekine, 1966). The CaATPase activities of myosin respond in different but characteristic ways to modification by NEM depending on which of the two sulfhydryls SH_1 or SH_2 is being modified. We have employed these differences in behavior as criteria

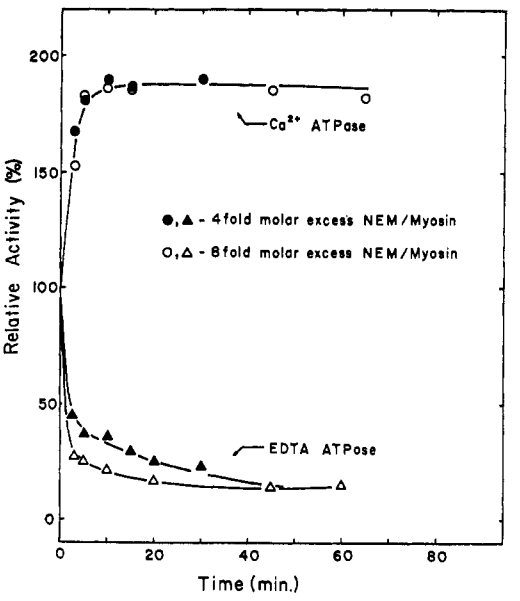

FIGURE 3: Relative Ca^{2+} and EDTA ATPases of myosin reacted at pH 7.9 with a fourfold and eightfold molar excess of NEM plotted as a function of time of modification. 100% activity corresponds to 3 μmol of P_i mg^{-1} min^{-1} for EDTA ATPase and to 1 μmol of P_i mg^{-1} min^{-1} for CaATPase.

for evaluating the influence of MgATP and MgADP on the course and extent of reaction of NEM with myosin.

Figures 3 and 4 show the influence of MgADP (10^{-3} M) on the course of reaction of myosin with NEM at pH 7.9. In the absence of MgADP (Figure 3) the reaction with NEM is clearly directed to SH_1, since the CaATPase activity is elevated and the EDTA ATPase activity is lost. Moreover, a comparison of the levels of activities obtained with fourfold and eightfold molar excess of NEM over myosin (Figure 3) demonstrates that a fourfold molar excess of NEM is sufficient to react completely with the SH_1 group. In the presence of MgADP, the same reaction between NEM and myosin has been significantly modified as seen in Figure 4. In this case, a rapid reaction apparently occurs at the SH_2 site only, since EDTA ATPase activity is lost while the CaATPase level remains unchanged. When the same reaction is performed at a twofold molar excess of NEM over myosin only partial loss (45%) of EDTA ATPase is observed. To verify that under these experimental conditions the reac-

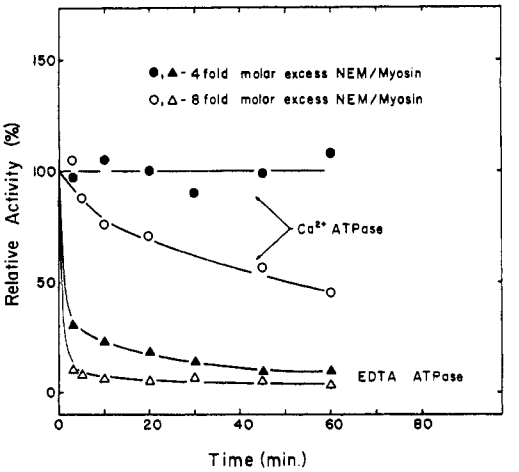

FIGURE 4: Relative Ca^{2+} and EDTA ATPase of myosin reacted at pH 7.9 in the presence of 10^{-3} M MgADP with a fourfold and eightfold molar excess of NEM.

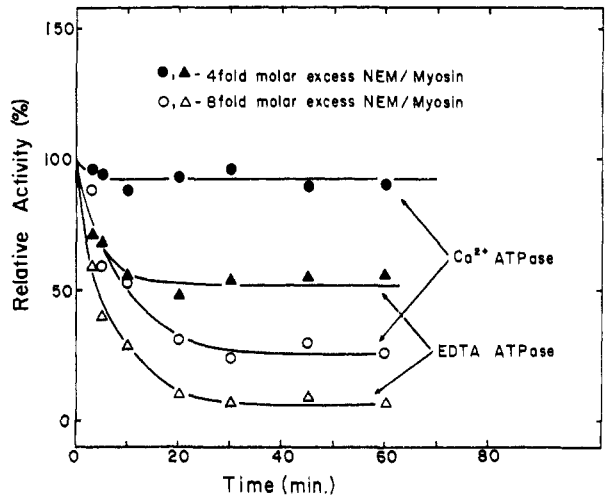

FIGURE 5: Relative Ca^{2+} and EDTA ATPases of myosin reacted at pH 7.9 in the presence of 10^{-3} M MgATP with a fourfold and eightfold molar excess of NEM.

tion with NEM proceeds at the SH_2 site, the reaction product was checked for ionic strength and temperature dependence of its CaATPase activity and was subjected to the NEM remodification test. The observed behavior was identical with that of the SH_2-NEM myosin obtained *via* reversible blockage of the SH_1 site with FDNB (see previous section).

Increasing the amount of NEM to a level of eightfold molar excess over myosin allows the reaction to occur at both SH_2 and SH_1, although at a significantly lower rate for the latter, as discerned from the gradual decrease in CaATPase activity in this case.

Substitution of MgATP for MgADP (10^{-3} M) provides protection for the SH_2 site. As shown in Figure 5, the EDTA ATPase activity decreases to only approximately 50% of the control without any discernible change in the CaATPase activity when a fourfold molar excess of NEM is employed. Under these conditions, as with MgADP, the SH_1 is fully protected and the partial modification which occurs in the presence of MgATP is localized at the SH_2 site. At a lower molar excess of NEM over myosin (twofold) MgATP affords

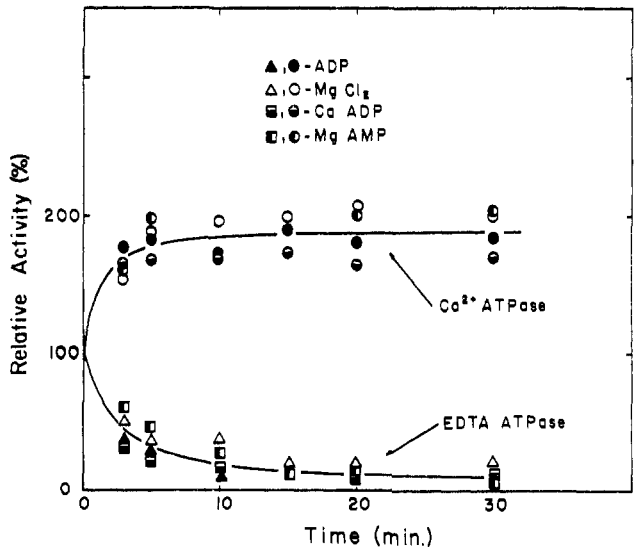

FIGURE 6: Relative Ca^{2+} and EDTA ATPases of myosin reacted at pH 7.9 with a fourfold molar excess of NEM. The ligands present in the reaction mixture were at a concentration of 10^{-3} M.

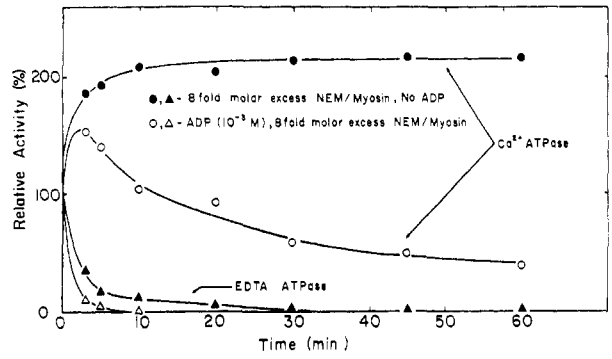

FIGURE 7: Relative Ca^{2+} and EDTA ATPases of myosin reacted at pH 7.9 with an eightfold molar excess of NEM in the presence and absence of 10^{-3} M ADP.

almost complete protection to both SH_1 and SH_2 (EDTA ATPase activity decreases only 15%). With a higher excess of NEM (eightfold) the reaction in the presence of MgATP apparently proceeds at both SH_2 and SH_1, although the reaction in the case of the latter is considerably slower. A similar behavior has been noted at pH 7.0 for both MgATP and MgADP except that here the binding interaction between MgADP and myosin is somewhat weaker since incomplete protection of SH_1 occurs. It should be noted that modification reactions by NEM described here were found to proceed in identical fashion when conducted at low ionic strength solvent (0.075 M KCl–0.01 M Tris, pH 7.9).

The above experiments support our contention that the inhibitory effect of MgATP is caused by a specific binding interaction involving both the SH_1 and SH_2 sulfhydryl sites. Furthermore, since the inhibition is apparently specific for the Mg^{2+} nucleotide complex, we would not expect Mg^{2+}, ADP, or Ca ADP to duplicate its effect on the reaction between NEM and myosin. This expectation has been confirmed by the experimental results depicted in Figure 6, where it is shown that the presence of each of these ligands individually has essentially no effect on the reaction between NEM and myosin at fourfold molar excess of the reagent over the protein. This is observed despite the reasonable supposition that the presence of ADP or CaADP in the reaction system should expose the SH_2 site, in analogy to their effect on the SH_1-NEM myosin. The accessibility of the SH_2 site in the presence of ADP can, however, be detected by increasing the amount of NEM in the reaction. Accordingly, Figure 7 demonstrates that at an eightfold molar excess of NEM to myosin the modification proceeds in the presence of ADP at both SH_1 and SH_2 as evidenced by a gradual decrease in the CaATPase activity. We have observed that the SH_2 group does not react with NEM in the presence of pyrophosphate orMg pyrophosphate suggesting that the adenine moiety is a necessary prerequisite for this function. We have also found that MgAMP has no effect on the reaction between NEM and myosin (Figure 6) which leads to the conclusion that the presence of the β and γ, *i.e.*, penultimate and terminal phosphoryl, groups is essential for the formation of the inhibitory structure involving Mg^{2+} nucleotide and the two sulfhydryl sites of myosin. Such an inhibitory structure is apparently not formed, however, with participation of β,γ-imino-ATP or α,β-methylene-ATP (at saturating concentrations) as their Mg complexes did not affect the modification reaction.

Discussion

At millimolar levels of MgATP the activity of myosin is activated following the selective modification by NEM of

either the SH_1 or SH_2 group. However, the same interaction when allowed to occur with both SH sites (in unmodified myosin) brings about strong inhibition of MgATP hydrolysis. We suggest (Burke *et al.*, 1973) that at MgATP levels sufficient to saturate the weak binding site, SH_2 (Sugden and Nihei, 1969), an inhibiting cyclic structure is formed between the two SH sites and MgATP. When the SH_1 site is modified by NEM the same saturating levels of MgATP, with respect to the SH_2 site, are required for expression of the elevated ATPase activity.

The proposed mechanism can be tested in a number of ways. It was considered likely, for example, that the loss of the inhibiting effect of MgATP following blockage of SH_1 is mainly due to the loss of the binding potential of this site with respect to Mg^{2+} or MgATP and not to some steric consequence of the modification. Thus it was anticipated that an SH_1-linked reagent which could be easily converted into a nucleophilic derivative should restore the inhibitory effect of MgATP upon such conversion. This expectation was verified experimentally by modification of the SH_1 site with FDNB following by reduction of SH_1-DNP myosin (with sodium dithionite) to yield SH_1-phenylenediamine myosin. As shown in Figure 2, SH_1-DNP myosin is activated at millimolar concentrations of Mg^{2+} ion. However, upon conversion of the DNP group to its reduced phenylenediamine derivative, which is known to coordinate with metal ions (Prasad and Reddy, 1958; Prasad *et al.*, 1960), the inhibiting effect of Mg^{2+} ions on the ATPase of myosin is restored, though it is shifted to higher Mg^{2+} concentrations (Figure 2). 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 CaATPase activity of the latter remains highly activated (Table I). It is likely that the structure of CaATP, as opposed to that of MgATP, precludes its interaction with the SH_1-phenylenediamine site, thus restricting the binding to the free SH_2 site. In more specific terms, the difference in the ATPase activity occurring on binding at millimolar concentrations of MgATP or CaATP to myosin probably reflects differences in the structures of these metal complexes and the chelation potential of the two divalent cations. In this connection it is known from crystallographic studies that Mg^{2+} and Ca^{2+} pyrophosphate complexes form boat conformations in which the charged oxygens are staggered and eclipsed, respectively (Mildvan, 1970). The requirement of both sulfhydryl sites for Mg^{2+} inhibition suggests that a cyclic structure (E⟲Mg substrate) may be responsible for the observed behavior. Substituting a phenylenediamine at the SH_1 site still retains the coordination potential of this site. The high activity levels of CaATPase and its requirement for only one sulfhydryl site indicate that an inhibiting cyclic structure cannot be formed between Ca^{2+} nucleotide complexes and the two sulfhydryl sites.

The above conclusions lead to certain predictions which again can be easily tested. Since inhibition is found only in the case of MgATP and not for CaATP (in the millimolar range) we would expect the binding interaction of MgATP (or MgADP) to be quite different from that of CaATP, CaADP, ADP, or Mg^{2+} alone. These differences should be reflected in the effects of the above ligands on the course of reaction of NEM with myosin.

The results presented in Figures 3–6 demonstrate that binding of MgADP or MgATP to myosin dramatically alters the reactivity of the sulfhydryl sites; the binding of the other ligands shows a negligible effect on the reaction with NEM. Under standard conditions of reaction between NEM and myosin (pH 7.9, no ligands present) the SH_1 group is easily modified whereas the SH_2 group remains buried and inaccessible. The reactivity of the SH_2 site was examined in detail by Yamaguchi and Sekine (1966) who found, using SH_1-NEM blocked myosin, that the presence of ADP, ATP, or their metal complexes is required to expose the SH_2 site for modification by NEM. In their study modification of the SH_2 group proceeded more rapidly in the presence of the metal–nucleotide complexes than in the presence of ADP or ATP. In the case of SH_1-NEM myosin it seems likely that the role of various nucleotides and their complexes is probably confined to producing local conformational changes or structural perturbations around the SH_2 site.

A different situation arises, however, when the modification by NEM proceeds with native myosin. ADP, CaADP, or $MgCl_2$ alone have no effect on this reaction (Figure 6) which at pH 7.9 and with a fourfold molar excess of NEM over myosin results in blocking of the SH_1 site. Even though the SH_2 site is apparently exposed in the presence of ADP or CaADP its reactivity is much lower than that of the SH_1 site allowing for a preferential modification of the latter. The evidence for exposure of the SH_2 site comes from the fact that increasing the molar excess of NEM over myosin to eightfold allows the reaction in the presence of ADP to occur both at SH_1 and SH_2 sites (Figure 7). Judging from the initial elevation of CaATP activity (Figure 7) the selective modification of SH_1 sites precedes the reaction with SH_2 sites. A similar pattern of modification of myosin by NEM in the presence of ADP was observed by Sekine and Yamaguchi (1963) and (at room temperature) by Daniel and Hartshorne (1973).

A specific and dramatic effect on the reaction of myosin with NEM is produced by binding of MgADP or MgATP to the protein. Binding of MgADP at pH 7.9 to unmodified myosin protects the SH_1 site from reaction with NEM while at the same time exposing the SH_2 site to reaction (Figure 4). Since no protection of SH_2 apparently occurs here, it is likely that MgADP does not interact directly with this sulfhydryl site. The reason no modification of SH_1 site can be detected is most probably due to the exhaustion of the reagent in competing, nonspecific reactions. Increasing the amount of NEM in the reaction to an eightfold molar excess over myosin allows effective competition with MgADP for the SH_1 site, thereby enabling modification of this site also.

A higher degree of protection of both SH sites is observed if MgADP is replaced by MgATP. In this case, at fourfold molar excess of NEM over myosin, in addition to full protection of the SH_1 site, the SH_2 site is partially protected as well (Figure 5). This result is not unexpected since MgATP is known to have a higher affinity for myosin than MgADP (Schliselfeld and Bárány, 1968; Malik *et al.*, 1972). Thus MgATP will form a more stable ternary cyclic complex with the two SH sites and consequently provide some protection for the weak binding site (SH_2). As in the case of MgADP, increasing the amount of NEM in the reaction allows this reagent to compete with MgATP for the SH sites and thus to modify them. As deduced from Figures 3 to 6, the specific binding interaction between the Mg nucleotides and the SH_1 and SH_2 sites of myosin apparently requires the β and possibly the γ phosphoryl groups, since MgAMP had no effect on the reaction of NEM with myosin.

These results may be compared to the studies of Bárány *et al.* (1969, 1972, and references therein) on the incorporation of FDNB and NEM into myosin. These workers demonstrated that the labeling of myosin is essentially confined to

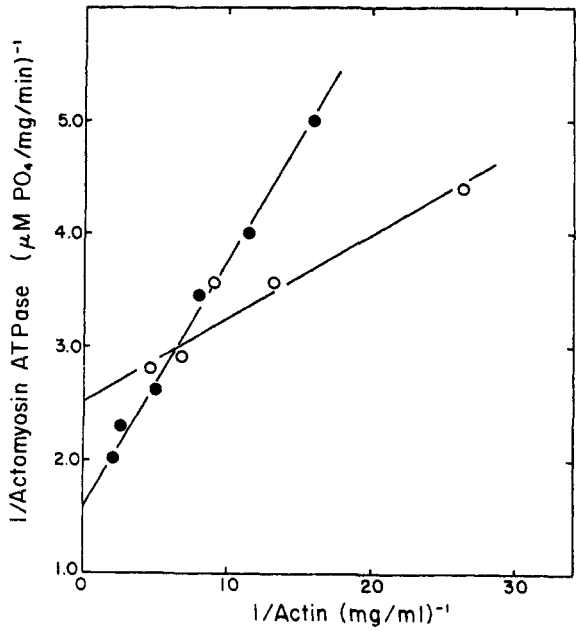

FIGURE 8: Reciprocal plots of ATPases of actomyosin (●) and acto-SH_1-NEM myosin (○) *vs.* actin concentration. Conditions are the same as Figure 2. Myosin concentration was 0.18 mg/ml.

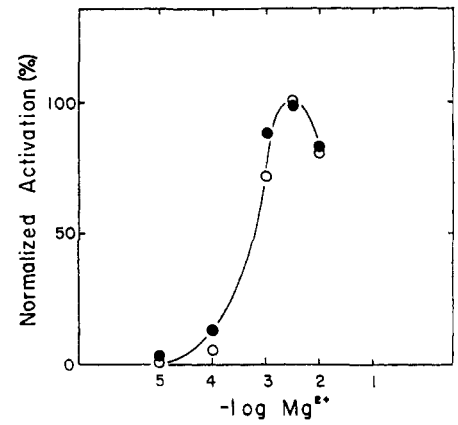

FIGURE 9: Normalized effect of added Mg^{2+} on activation of ATPase activity of myosin by actin (●) or by modification of the SH sites with NEM (○). Conditions are the same as in Figure 2. Activation by actin is defined as the ratio of ATPase of actomyosin (extrapolated to infinite concentration of actin) to that of myosin at a given Mg^{2+} concentration. Activation on modification is defined as the ratio of ATPase of SH_1-NEM myosin to that of myosin at a given Mg^{2+} concentration. 100% activation by actin corresponds to 45-fold activation and for SH_1-NEM to eightfold activation (over the ATPase of unmodified myosin at 5 mM Mg^{2+}).

its globular "head" region and they have used this technique to detect conformationally induced changes in the accessibility (and therefore reactivity) of the essential sulfhydryl groups of myosin in the presence of various ligands. Whereas the presence of $MgCl_2$, $CaCl_2$, MgADP, or MgAMP was found to have no effect on the amount of NEM or FDNB incorporated into myosin, a marked reduction in the incorporation of these labels was observed in the presence of MgATP. The last result demonstrates that the strong interaction of MgATP with the two essential sulfhydryl sites of myosin reduces the reactivity of these sites to NEM. However, the invariant incorporation of FDNB or NEM into myosin in the presence of the other ligands does not necessarily imply that the binding of these ligands has no effect on the modification reaction. Concomitant protection of a reactive sulfhydryl group (SH_1) and the exposure of a formerly nonreactive and buried sulfhydryl group (SH_2) can occur, resulting in the same extent of incorporation of label. This applies particularly to the case of MgADP binding to myosin since our results indicate that in the presence of this ligand the SH_1 group is protected from reaction whereas the SH_2 group is exposed and is readily modified with NEM.

The results of Malik and Martonosi (1972) also reflect the very specific interaction between MgATP (and MgADP) and the sulfhydryl sites of myosin. These investigators have found that the uv difference spectrum at 288 mμ accompanying binding of the above ligands to the native myosin is not observed when these ligands are allowed to bind to NEM modified HMM. Apparently, modification of the sulfhydryl sites with NEM blocks the specific interactions responsible for the observed spectral changes on binding of these ligands (see also Werber *et al.*, 1972).

It is worth noting in this context that the involvement of sulfhydryl groups in the binding of metal containing substrates according to the scheme proposed in the present study is by no means unique. For example, Cohn and her coworkers (O'Sullivan *et al.*, 1966) have shown from the effect of Mg^{2+} on the reactivity of the two essential sulfhydryl groups in creatine kinase that only in the case of the MgATP–enzyme–creatine complex was full protection afforded to these sulfhydryl groups from reaction with iodoacetic acid or iodoacetamide.

The results presented in this and in the previous communication (Burke *et al.*, 1973) suggest that the high concentrations of Mg^{2+} ions in muscle are of regulatory importance; they are required at these levels (in the form of MgATP) to saturate the weak affinity site (SH_2) and thus form with the participation of both sulfhydryl sites, SH_1 and SH_2, the inhibiting cyclic complex. Modification by NEM of either one of these sites prevents the formation of such a structure and consequently abolishes the inhibition of myosin ATPase by Mg^{2+} ions. Actin may function in a similar way (Burke *et al.*, 1973). On binding to myosin it may mask one of the two sulfhydryl sites, thereby breaking the inhibiting cyclic structure and leading to a large activation of the ATPase activity of myosin.

We have shown previously that the activation profiles of the MgATPase activity by actin (at 4:1 molar ratio of actin to myosin) and by NEM modification are identical over a large span of Mg^{2+} ion concentration. In order to substantiate this finding and to verify that it is not a fortuitous result of two competitive effects (activation by actin and dissociation of actomyosin by MgATP) we have extended our measurements to cover a wide range of actin to myosin ratios. This enabled us to construct reciprocal plots of 1/ATPase *vs.* 1/concentration of actin and to determine for each concentration of added Mg^{2+} ions the activation of ATPase activity at infinite excess of actin over myosin. The reciprocal plots for both unmodified and SH_1-NEM myosin are linear (Figure 8) as previously described for HMM and subfragment 1 by Eisenberg *et al.* (1968; Eisenberg and Moos, 1968). As shown in Figure 9, the normalized activation of ATPase activity at infinite actin concentrations displays the same profile with respect to concentration of added Mg^{2+} ion as that of the normalized activation induced by NEM modification of the SH_1 site. This by no means implies that in quantitative terms the two effects are identical. Such an identity could hardly be expected for effectors as different as actin and NEM when even simple sulfhydryl reagents differ markedly as to the magnitude of the induced effects (Table I,

rows 2 and 10). Evidently changes in the active patch of myosin accompanying the interaction of various effectors with SH_1 sites determine to a large extent the quantitative aspects of the observed activation or inhibition. As a primitive demonstration of differences between the effects of NEM and actin it should be noted that SH_1-NEM modified myosin can be further activated (3.5-fold) by infinite actin concentrations. The combined activation of myosin MgATPase due to modification of SH_1 by NEM (eightfold) and infinite actin excess (3.5-fold) is 28-fold, which is not dramatically different from the 45-fold activation of MgATPase of native myosin by infinite actin excess. We cannot equate the effects of NEM and actin on myosin, but the identity of their normalized activation profiles (Figure 9) suggests that in terms of inducing the activation of MgATPase of myosin they are similar in nature. Thus the conclusion that on binding to myosin actin breaks the inhibiting complex structure and perturbs the SH_1 site also seems to be valid under conditions of minimum dissociation of the actomyosin complex.

Several additional lines of evidence indicate that the binding of actin affects the sulfhydryl sites of myosin. Studies on incorporation of NEM or FDNB into actomyosin show that actin protects the SH sites from modification (Bárány *et al.*, 1969; Schaub and Watterson, 1972). This protection is afforded mainly to the SH_1 site which, in the absence of actin and under reaction conditions employed by Bárány *et al.* (1969), is the site of modification. The same conclusion may be derived from the work of Schaub and Watterson (1972). Recently, Seidel (1973) has shown that on binding to myosin previously modified with a spin-label at either SH_1 or SH_2 site, actin perturbs only the label attached to the SH_1 site. This again indicates that actin does in fact interact with the SH_1 site. An analogy can also be drawn between the temperature dependence of the MgATPase activity of acto-HMM and that of SH_1-NEM myosin. For unmodified myosin the temperature dependence of its MgATPase is extremely low[2] (fourfold increase over the range of 10–35°). However, both in the case of SH_1NEM myosin[2] and acto-HMM (Barouch and Moos, 1971) temperature has a dramatic effect on their activity (35-fold increase over the range of 10–35°). Altogether, the evidence so far accumulated strongly implicates the SH_1 site in the interaction of myosin with actin. This interaction is responsible for disruption of the inhibiting cyclic structure, between MgATP and the two sulfhydryl sites, and is, we believe, an integral part of the contractile cycle regulatory mechanism. When trying to extend this conclusion to the acto-HMM or acto-subfragment I systems we encounter a number of difficulties. The activation of MgATPase of HMM at low ionic strength (0.02–0.08 M) due to modification of SH_1 by NEM is only two- to threefold whereas the activation by actin is about 200-fold (Silverman *et al.*, 1972). This large difference is not narrowed significantly by interaction of actin with SH_1 blocked HMM (threefold activation, Silverman *et al.*, 1972). A similar discrepancy exists in the acto-subfragment I system in spite of the fact that the activation profiles (by NEM or actin) when examined as a function of Mg^{2+} concentration display behavior identical with that observed for the myosin system (M. Burke, E. Reisler, and W. F. Harrington, unpublished data). However, it should be recognized that the degree of activation of these proteolytic fragments by actin at physiological ionic strengths (0.15 M KCl) is markedly lower (*ca.* tenfold; Hozumi and Tawada, 1973). It is possible that preparative procedures involving enzymatic digestion of myosin to prepare HMM or subfragment I can modify the interaction of the resulting products with actin due to the proteolytic cleavages introduced into their heavy- and light-chain regions. This may occur even though the interactions with simple substrate moleclues are apparently unchanged as evidenced by normal activity values. In fact, it should be noted that the affinity of actin for HMM or subfragment I is smaller than for myosin. At comparable experimental conditions HMM and subfragment I are activated fivefold and twofold, respectively, by actin (Eisenberg *et al.*, 1968) compared with 25-fold activation of myosin (Burke *et al.*, 1973). At infinite actin excess this pattern is reversed with HMM and subfragment I being activated 200-fold and myosin 45-fold. The ionic strength dependence of the interactions between actin and myosin and proteolytic fragments of myosin is also markedly different. Thus, any comparison between the three systems, actomyosin, acto-HMM, and acto-subfragment I, should be made under identical ionic strength conditions. Obviously, these conditions should correspond to the physiological state if pitfalls of misinterpretation are to be avoided. It appears that at very low ionic strength conditions interactions not directly related to the Ca^{2+} regulated contractile process can occur (Figure 2 in Gordon *et al.*, 1973). Additional complexity is introduced into the comparison of acto-HMM with actomyosin by differences in their structural organization. Myosin particles associate at low ionic strength conditions to form filaments which resemble the physiological structures, whereas HMM remains in the dispersed state. The state of aggregation of myosin may in turn have a direct bearing on the possible cooperative nature of actomyosin interactions. Considering all these effects, we believe that direct extrapolation of results obtained on the actomyosin system to acto-HMM or acto-subfragment I systems should be treated with caution.

It is of some interest to speculate on the relationship between the activation scheme presented above and the recent kinetic mechanism of ATP hydrolysis developed by Taylor and his coworkers (Finlayson *et al.*, 1969; Lymn and Taylor, 1971) and Trentham *et al.* (1972). Lymn and Taylor (1971) have shown that the dissociation of acto-HMM upon binding of MgATP occurs under physiological conditions at a rate at least ten times faster than the rate of splitting of ATP. They conclude that hydrolysis in living muscle occurs predominantly when ATP is in the myosin–MgATP state. Actin reacts with the resulting myosin·MgADP·P_i complex leading to rapid product release. We believe that MgATP, bound in a very stable cyclic complex involving the SH_1 and SH_2 groups, competes effectively with actin for the SH_1 site. Furthermore, we speculate that the hydrolysis step lowers the stability of the cyclic complex through formation of the "myosin–product complex" (in whatever intermediate form it may exist) allowing actin to recombine with the SH_1 site. We are encouraged to speculate along these lines in view of our modification experiments, which demonstrate that MgADP renders less protection than does MgATP against modification of the SH sites by NEM suggesting, in turn, that the MgADP–myosin complex is less stable than the ternary MgATP–myosin cyclic complex.

The relationship between the early burst phenomenon and our findings is also of some interest. As shown by Lymn and Taylor (1971) the first mole of ATP is hydrolyzed at a rate (150 sec^{-1}) considerably faster than the steady-state rate. The early burst is eliminated in the presence of EDTA but hydroly-

[2] Unpublished results. Similar magnitudes of temperature dependence were observed for the CaATPase of unmodified and SH_1-NEM modified myosin, respectively (Sekine and Kielley, 1964).

sis proceeds (in 0.5 M KCl, pH 8, 20°) at a markedly higher steady-state rate than that observed with Ca^{2+} or Mg^{2+} ATP. Again, we speculate that the early burst is directly related to the presence of a chelate structure involving a divalent metal ion (Mg^{2+} or Ca^{2+}) and the neighboring phosphate oxygen atoms of the substrate. The first hydrolytic step cleaves the terminal phosphate, but this group as well as the ADP moiety are firmly held by strong coordination linkages to the Mg^{2+} (or Ca^{2+}) ion resulting in a slow decay of the resulting intermediate complex. In the presence of EDTA the divalent metal concentration is reduced to a level too low for chelate formation with the substrate. In this case the ADP and PO_4 moieties are no longer firmly linked through the metal ion and, following hydrolysis, the split product intermediate can undergo rapid decay. Thus the bond hydrolysis step can become rate limiting.

We should emphasize that in the burst phenomenon we are focusing attention on the segment of the overall chelate structure formed between metal and substrate. The coordination linkages of the complex to the SH_1 and SH_2 sites play their role in binding the substrates within the active site region.

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Added in Proof

Watterson and Schaub (1973) have recently reported that the rate of inactivation of myosin by NEM is the same in the presence of MgADP + P_i and MgADP but is considerably slower in the presence of MgATP. It was concluded in agreement with our findings that the conformation of the myosin–MgATP complex differs from that of the myosin–product complex.

References

Bailin, G., and Bárány, M. (1972a), *J. Biol. Chem. 247*, 7815.

Bailin, G., and Bárány, M. (1972b), *J. Mechanochem. Cell Motility 1*, 189.

Bárány, M., Bailin, G., and Bárány, K. (1969), *J. Biol. Chem. 244*, 648.

Bárány, M., and Bárány, K. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37*, 157.

Barouch, W. W., and Moos, C. (1971), *Biochim. Biophys. Acta 234*, 183.

Burke, M., Reisler, E., and Harrington, W. F. (1973), *Proc. Nat. Acad. Sci. U. S. 70*, 3793.

Daniel, J. L., and Hartshorne, D. J. (1973), private communication.

Eisenberg, E., and Moos, C. (1968), *Biochemistry 7*, 1486.

Eisenberg, E., Zobel, C. R., and Moos, C. (1968), *Biochemistry 7*, 3186.

Finlayson, B., Lymn, R. W., and Taylor, E. W. (1969), *Biochemistry 8*, 811.

Gazith, J., Himmelfarb, S., and Harrington, W. F. (1970), *J. Biol. Chem. 245*, 15.

Godfrey, J. E., and Harrington, W. F. (1970), *Biochemistry 9*, 886.

Gordon, A. M., Godt, R. E., Donaldson, S. K. B., and Harris, C. E. (1973), *J. Gen. Physiol. 62*, 550.

Gratzer, W., and Lowey, S. (1969), *J. Biol. Chem. 244*, 22.

Hozumi, T., and Tawada, K. (1973), *J. Biochem. (Tokyo) 74*, 385.

Kielley, W. W., and Bradley, L. B. (1956), *J. Biol. Chem. 208*, 653.

Kielley, W. W., Kalckar, H. M., and Bradley, L. B. (1956), *J. Biol. Chem. 219*, 95.

Lymn, R. W., and Taylor, E. W. (1971), *Biochemistry 10*, 4617.

Malik, M. N., Marchioli, L., and Martonosi, A. (1972), *Arch. Biochem. Biophys. 153*, 147.

Malik, M. N., and Martonosi, A. (1972), *Arch. Biochem. Biophys. 152*, 243.

Mildvan, A. S. (1970), *Enzymes, 3rd Ed. 2*, 446.

O'Sullivan, W. J., Diefenbach, H., and Cohn, M. (1966), *Biochemistry 5*, 2666.

Prasad, S., Krishnaiah, K. S. R., and Ranganathachar, T. (1960), *J. Indian Chem. Soc. 37*, 344.

Prasad, S., and Reddy, V. R. (1958), *J. Indian Chem. Soc. 35*, 722.

Schaub, M. C., and Watterson, J. G. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37*, 153.

Schliselfeld, L. H., and Bárány, M. (1968), *Biochemistry 7*, 3206.

Seidel, J. C. (1969a), *J. Biol. Chem. 244*, 1142.

Seidel, J. C. (1969b), *Biochim. Biophys. Acta 180*, 216.

Seidel, J. C. (1973), *Arch. Biochem. Biophys. 157*, 588.

Seidel, J. C., and Gergely, J. (1971), *Biochem. Biophys. Res. Commun. 44*, 826.

Sekine, T., Barnett, L. M., and Kielley, W. W. (1962), *J. Biol. Chem. 237*, 2769.

Sekine, T., and Kielley, W. W. (1964), *Biochim. Biophys. Acta 81*, 336.

Sekine, T., and Yamaguchi, M. (1963), *J. Biochem. (Tokyo) 54*, 196.

Shaltiel, S. (1967), *Biochem. Biophys. Res. Commun. 29*, 178.

Shaltiel, S., and Soria, M. (1969), *Biochemistry 8*, 4411.

Silverman, R., Eisenberg, E., and Kielley, W. W. (1972), *Nature (London) 240*, 207.

Sugden, E. A., and Nihei, T. (1969), *Biochem. J. 113*, 821.

Trentham, D. R., Bardsley, R. G., Eccelston, J. F., and Weeds, A. G. (1972), *Biochem. J. 126*, 635.

Warren, J. C., Stowring, L., and Morales, M. F. (1966), *J. Biol. Chem. 241*, 309.

Watterson, J. G., and Schaub, M. C. (1973), *Hoppe-Seyler's Z. Physiol. Chem. 354*, 1619.

Werber, M. M., Szent-Györgyi, A. G., and Fasman, G. D. (1972), *Biochemistry 11*, 2872.

Yamaguchi, M., and Sekine, T. (1966), *J. Biochem. (Tokyo) 59*, 24.

Yoshino, H., Morita, F., and Yagi, K. (1972), *J. Biochem. (Tokyo) 71*, 351.