Myosin ATP Hydrolysis: A Mechanism Involving a Magnesium Chelate Complex

(MUSCLE/SULFHYDRYL SITES/ATPASE/ACTIN)

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ABSTRACT It is suggested that under physiological conditions (> 1 mM Mg++), MgATP binds to myosin to form a chelate involving the two reactive sulfhydryl sites (SH1 and SH2). The stability of the chelate structure results in marked inhibition of the myosin ATPase in the presence of millimolar magnesium ion. The inhibitory effect of magnesium ion can be eliminated chemically by blocking either the SH1 or SH2 site since this precludes formation of the chelate. In muscle, actin apparently behaves in a similar fashion in that its interaction with myosin causes a disruption of the chelate structure.

It has been known for many years that the ATPase activity of myosin is maximally activated by Ca++ or in the presence of the divalent metal chelator, EDTA, whereas it is strongly inhibited by Mg++ at concentrations in the millimolar range (1-3). The fundamental reasons for this behavior have remained obscure, but a large number of observations has related the ATP cleavage reaction to an involvement of one or possibly two cysteine residues within or near the active-site region. Blockage of one sulfhydryl group per subunit (SH1) was shown to elevate the Ca++ ATPase activity about 4-fold while causing a concomitant loss in the EDTA-ATPase (3) and in the ability of actin to activate (4). Subsequent blocking of a second thiol group per subunit (SH2) results in complete loss of myosin ATPase activity (5).

With this information as background and in light of the experimental results to be presented below, we wish to propose a novel scheme of ATP cleavage by myosin which, we believe, can provide a self-consistent and unifying explanation for these diverse phenomena. The scheme strongly implicates the closing and opening of a metal chelate complex involving MgATP and the two SH groups mentioned above as fundamental to the resting and active state of muscle.

We propose that MgATP on addition to myosin forms a stable chelate structure involving the two sulfhydryl sites, SH1 and SH2++. This structure is rapidly cleaved at the terminal phosphate to give the early burst. The resulting myosin-product complex remains in the form of a stable chelate structure. In consequence, its dissociation becomes rate limiting and thus responsible for the low rate of ATP hydrolysis by myosin in the presence of Mg++ ions. Blocking of either the SH1 or SH2 site prevents formation of a chelate structure. Here MgATP, and consequently also the products, are linked to myosin through one of the SH sites. This linkage results in much faster dissociation of the myosin-product complex and a high rate of ATP hydrolysis.

As we shall demonstrate, when the SH1 site of myosin is blocked, Mg++ and Ca++ ATPases show a similar type of dependence on the concentration of the respective cation. They share this behavior in common with the Mg++ ATPase of actin-activated myosin, suggesting that binding of actin to myosin results in a rapid dissociation of the product complex from the SH1 site. Thus we believe that the activating effect of actin is caused by its binding to the subfragment-I moiety of myosin in the vicinity of the SH1 site. This process is responsible for disruption of the stable chelate form of the myosin-product complex and subsequent rapid release of the products from the weak binding SH2 site.

The high rate of ATP hydrolysis by myosin in the presence of millimolar Ca++ (no Mg++) results from the inability of CaATP to form a chelate structure through the two SH sites. When the SH1 site of myosin is blocked, hydrolysis of CaATP occurs with participation of the SH2 site but the reduced binding affinity of this substrate to the SH2 site results in higher turnover rate for the hydrolytic cycle.

In the case of rapid cleavage of ATP in the absence of divalent cations, our results demonstrate that both SH1 and SH2 sites are obligatory participants in the reaction. The primary activating effect of EDTA is to lower the Mg++ ion concentration to a level well below that required for chelate formation. Thus, in the presence of EDTA the absence of an early burst can be attributed to lack of divalent cation (M++) resulting in a rapid steady-state reaction.

MATERIALS AND METHODS

ATP of highest available purity was purchased from Sigma Chemical Company, St. Louis, Mo. Inorganic salts and reagents were analytical grade. The SH1 groups of myosin were reversibly dinitrophenylated by the method of Bailin and Bárány (6). Optimal results were obtained by reacting the protein (2 mg/ml) with a four-fold molar excess of reagent at pH 8.3 for 40 mins at 4°C. Selective modification of the SH1 groups with N-ethylmaleimide (NEM) was achieved by the method of Sekine and Kielley (3). Selective modification of SH2 groups was obtained by prior dinitrophenylation of SH1 and subsequent reaction of SH2 groups of the modified myosin with NEM as described by Yamaguchi and Sekine (5). The SH2 groups were regenerated by subsequent thiolysis with 2-mercaptoethanol (6). Reduction to the dinitrophenyl group to a phenylenediamine derivative was achieved with

Abbreviations: NEM, N-ethylmaleimide; FDNB, fluorodinitrobenzene.

* The term "chelate" in this context does not distinguish between binding types, protein-metal-substrate, or protein-substrate-metal. It signifies a cyclic ternary complex.

† In the present context the term "myosin-product complex" refers to whatever intermediate forms of this complex may exist; it distinguishes them from the preceding myosin-substrate complex and the final dissociation products.

3798
sodium dithionite at pH 8.0, using a 100-fold molar excess of reagent and reacting for 5–10 min at room temperature.

**RESULTS AND DISCUSSION**

The inhibition of myosin ATPase by Mg\(^{2+}\) ion, though well documented (7–10), has so far found no satisfactory explanation in spite of its obvious importance in muscle contraction and the large interest it commands (11, 12).

Fig. 1 shows the ATPase activity of myosin as a function of concentration of Mg\(^{2+}\) added to the reaction mixture. Inhibition of the enzymatic activity falls in the same range of Mg\(^{2+}\) concentration as that reported by Sugden and Nihel (10). It was postulated in the introduction that inhibition occurs due to formation of a chelate structure involving the two SH sites of myosin, Mg\(^{2+}\) and the substrate. Sugden and Nihel (10) have shown that these two SH sites are important in metal binding, each site yielding a different type of binding reaction between Ca\(^{2+}\) and myosin. The SH\(_1\) site has a high affinity for Ca\(^{2+}\) whereas the SH\(_2\) site shows only a weak affinity for this metal. These authors could not observe strong binding of Ca\(^{2+}\) when the SH\(_1\) site was reactive with NEM. The weak binding of Ca\(^{2+}\) to the SH\(_2\) site, which was unaffected in this modification, was tentatively associated with the activation of CaATPase (10). Binding of Mg\(^{2+}\) to myosin follows the same pattern as that described for Ca\(^{2+}\) (11, 13), though the kinetic consequences of binding of the two cations are diametrically different. It will be noted that under our experimental conditions full inhibition of ATPase activity by Mg\(^{2+}\) is achieved (Fig. 1) at 10\(^{-4}\) to 10\(^{-3}\) M Mg\(^{2+}\). We believe that saturation of the weak binding site for Mg\(^{2+}\) (identified with SH\(_2\)) occurs in this concentration range and it is this saturation that allows formation of the inhibiting chelate structure. If formation of such a chelate, as postulated here, indeed requires participation of both the strong and the weak metal-binding sites, then its formation should be prevented by blocking Mg\(^{2+}\) binding to either one of these sites. Modification of the SH\(_2\) site with NEM, which eliminates the strong binding of Mg\(^{2+}\) (or Ca\(^{2+}\) to myosin, should therefore eliminate ATPase inhibition at high levels of Mg\(^{2+}\). Moreover, since only one binding site is available in the modified myosin for MgATP, the hydrolysis should be activated at Mg\(^{2+}\) levels that saturate this site. The activation of SH\(_2\)-NEM-modified myosin is indeed initiated at 10\(^{-4}\) M Mg\(^{2+}\) (Fig. 1), which according to the inhibition curve, is the saturating concentration for the weak binding site. It is important to note that when the inhibiting chelate structure cannot be formed (i.e., when SH\(_1\) is blocked), the ATPase activity of myosin reaches its highest value at physiological levels of Mg\(^{2+}\) ion. At 5 mM Mg\(^{2+}\), the SH\(_2\)-NEM-modified myosin is activated eight-fold over unmodified myosin.

The weak binding property of the SH\(_2\) site, though it demands high levels of metal ion for saturation, allows reversibly, for a rapid hydrolysis rate. It appears that even blocking of SH\(_1\) only the weak SH\(_2\) site is involved in the binding and hydrolysis of the metal substrate.

In absolute terms, the ATPase of unmodified myosin at Mg\(^{2+}\) <10\(^{-4}\) M is significantly higher than that of the SH\(_2\)-NEM-modified protein at Mg\(^{2+}\) >10\(^{-4}\) M. The high ATPase of unmodified myosin at low concentrations of Mg\(^{2+}\) can be viewed as resulting from binding of (K\(^{+}\)) ATP rather than its divalent metal complex, which does not form at these low Mg\(^{2+}\) levels (10). It is expected that (K\(^{+}\)) ATP should be hydrolyzed at higher rates than MgATP because of its lower affinity for myosin (11).

The effect of modification of the SH\(_1\) site of myosin by NEM can be simulated qualitatively by blocking this site with fluorodinitrobenzene (FDNB) (6). Though the resulting Mg\(^{2+}\) activation is not as high as in the case of NEM, FDNB modification offers the advantage of an easily reversible reaction (6, 14) and we hoped that reduction of the nitro groups (to amino groups) in the FDNB label attached to the SH\(_1\)

\[\text{FDNB} \rightarrow \text{FDN} \]

† The participation of such a structure in formation of chelates has been shown in several cases (15, 16).
site might restore, at least partially, the coordination properties of this site. This hypothesis was confirmed experimentally. Modification of the SH$_1$ site of myosin with FDNB activates the MgATPase (at 5 mM Mg$^{2+}$) four-fold (0.05 μmol of phosphate/mg per min) over that of the unmodified myosin (0.012 μmol of phosphate/mg per min, determined in the pH stat at 25°C). Subsequent reduction of the attached label to its amino form restores the Mg$^{2+}$ inhibition to the same level as in the unmodified protein; i.e., it allows for reformation of the inhibiting chelate structure.

The significance of the modification of the SH$_1$ site and its effect on ATPase activity is important insofar as it clarifies the mechanism of contraction.

Clearly the function which we attribute to this modification, namely that of preventing the formation of a Mg$^{2+}$ chelate structure, has to be carried out under physiological conditions by another factor. The obvious choice is actin, which is well known as an activator of the ATPase activity of myosin. Therefore we postulate that actin acts to block the SH$_1$ site (possibly by binding in the vicinity of this site) and thus break the chelate or prevent its formation.

Fig. 2 shows the actin activation of myosin ATPase as a function of Mg$^{2+}$ concentration. Activation occurs over an identical range of Mg$^{2+}$ concentration as observed for SH$_1$-modified myosin in the absence of actin. That is, at physiological concentrations of Mg$^{2+}$, where actin exerts its maximum inhibiting effect on myosin ATPase, either actin or chemical blocking of the SH$_1$ site can induce ATPase activation. It was previously known (18) that the ATPase of actomyosin is highest at millimolar levels of Mg$^{2+}$, but these findings have not been analyzed in terms of the activating capacity of actin. Fig. 2 strongly suggests that chemical modification of the SH$_1$ site produces the same qualitative effect as actin in accelerating the rate of ATP hydrolysis. On covering of the SH$_1$ site by actin, the chelate structure is broken and very rapid dissociation of the split products occurs from the weak binding SH$_1$ site. Thus rapid completion of the hydrolytic cycle occurs through subsequent formation of the chelate structure and dissociation of actin by the incoming MgATP.

In quantitative terms the activation achieved by modification of the SH$_1$ site is smaller than that obtained with actin. SH$_1$-NEM myosin is subject to a further residual activation by actin (2.5 fold), which brings the enzymatic activities of actomyosin and acto-SH$_1$-NEM into closer agreement.

At Mg$^{2+}$ concentrations too low to favor formation of a chelate structure, actin has no activating function (see Fig. 2). In fact, as has been shown earlier, ATP hydrolysis by actomyosin is strongly inhibited (19) in the presence of EDTA.

Since we have involved both SH$_1$ and SH$_2$ sites in the chelate structure, it was of interest to examine the properties of myosin with only the SH$_2$ site modified by NEM. In Fig. 3 we compare the ATPase activity, as a function of Mg$^{2+}$ concentration, of SH$_2$-modified myosin with that of unmodified and SH$_1$-modified myosin. The small rise in relative ATPase activity of SH$_2$-modified protein occurs again at high Mg$^{2+}$ levels; the absolute ATPase activity, however, is lower than in SH$_1$-modified protein. This finding is easily understood if we recall that in SH$_1$-modified myosin, binding of the MgATP complex involves only the SH$_1$ site which, because it is a strong binding site (10), yields a low turnover rate for ATP hydrolysis. For the same reason, SH$_2$-modified myosin is activated at appreciably lower concentrations of Mg$^{2+}$ (Fig. 4). The residual ATPase that can be observed in SH$_2$-NEM myosin even at low Mg$^{2+}$ concentrations is attributed to incomplete blocking of this site. The magnitude of ATPase activation and its dependence on Mg$^{2+}$ concentration strongly suggest that SH$_1$ is the site affected by actin.

Examination of Fig. 3 reveals also that modification of either SH$_1$ or SH$_2$ eliminates ATPase activity at very low Mg concentrations. At these concentrations (10) the hydrolytic reaction apparently proceeds with (K$^+$) ATP. This conclusion is supported by the results of Table 1, which demonstrate that in the complete absence of free divalent metal (presence of EDTA) both sulfhydryl groups are required for hydrolysis of (K$^+$) ATP (see also ref. 20).

The known activating function of EDTA, which derives from removal of metal ions, primarily Mg$^{2+}$ (7, 8), results from a situation in which both SH sites are free to accept the
linking and cleavage of \((K^+)\) ATP. If only one site is available for \((K^+)\) ATP, no cleavage occurs (Table 1), though a \(M^+\)-substrate can still be hydrolyzed. The lack of \((K^+)\) ATP hydrolysis in the modified myosin implies that both SH sites have to participate in cleavage of \((K^+)\) ATP.

A different situation exists in the case of \(Ca^++\)-activated ATPase of myosin (at millimolar concentrations of \(Ca^+\) ion). As shown in Table 1, and as originally demonstrated by Sekine and Kielley (3), modification of the SH group by NEM results in an activation of CaATP. Surprisingly, however, modification of the SH group by NEM hardly affects the ATPase activity compared to unmodified myosin. These results can now be understood in terms of our proposed mechanism of ATP hydrolysis. In the unmodified protein the CaATP substrate interacts with the strong binding site (SH) and is cleaved at the normal rate. On modification of the SH group the CaATP hydrolysis involves only the weak binding site (SH) and, thus, the turnover rate for hydrolysis is higher. Modification of the SH group leaves the strong binding site (SH) apparently unaltered, and the reaction that takes place involves only the participation of the SH1 site. The important outcome of this interpretation is that \(Mg^++\) is able to form an inhibiting chelate structure involving ATP and the two SH sites whereas \(Ca^+\) ions cannot form such a structure. The CaATP substrate can be cleaved on blocking of either SH site. This explanation accounts for the large differences in the rate of ATP hydrolysis in the presence of millimolar \(Mg^++\) or \(Ca^+\) ions.

The stability of the inhibiting chelate structure formed at physiological concentrations of \(Mg^++\) ion can be demonstrated by ATPase activity measurements. Myosin ATPase in the presence of both \(Mg^++\) and \(Ca^+\) ions in the millimolar range shows almost no activation compared to that in the presence of \(Mg^++\) alone (Table 2). Apparently the chelate structure formed by \(Mg^++\) cannot be opened by high \(Ca^+\) concentrations, even at values much above the physiological level of this cation. However, when the formation of a chelate structure is prevented by modification of the SH1 site, addition of \(Ca^+\) ions to a \(Mg^++\)-myosin system markedly elevates the ATPase activity (Table 2) in spite of the fact that MgATP binds much more strongly to SH sites than CaATP.

Results presented in Table 2 support the ideas developed in this contribution on the mechanism of ATP hydrolysis. They also render support to the current view that \(Ca^+\) has no direct regulatory control over the reaction of vertebrate myosin with MgATP. Its function appears to be limited to a crucial, but different, role in muscular contraction involving an indirect regulation of the myosin–actin interaction.

\(§\ Mg^++\) is known to have a strong tendency to form chelate structures involving ATP or ADP and some proteins, whereas \(Ca^+\) ion is an extremely poor chelator (e.g., ref. 23).

### Table 1. Relative ATPase activities

<table>
<thead>
<tr>
<th></th>
<th>EDTA</th>
<th>Ca</th>
</tr>
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<tbody>
<tr>
<td>Myosin</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SH-NEM myosin</td>
<td>10</td>
<td>250</td>
</tr>
<tr>
<td>SH+NEM myosin</td>
<td>15</td>
<td>117</td>
</tr>
</tbody>
</table>

Activity was measured at 37°C (21, 22). 100% Ca ATPase refers to 0.97 µmol of Pi/mg per min in 0.05 M KCl, pH 7.6. 100% EDTA ATPase refers to 3 µmol of Pi/mg per min in 0.8 M KCl, pH 7.6.

### Table 2. ATPase activities in µmol of Pi/mg per min

<table>
<thead>
<tr>
<th></th>
<th>Mg, mM</th>
<th>Ca, mM</th>
<th>(Ca + Mg), mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin</td>
<td>0.015</td>
<td>0.70</td>
<td>0.020</td>
</tr>
<tr>
<td>SH-NEM myosin</td>
<td>0.090</td>
<td>0.60</td>
<td>0.191</td>
</tr>
</tbody>
</table>

ATP measurements were done at 25°C in 75 mM KCl, 1 mM ATP, pH 7.9 with an automatic pH-stat unit.

### CONCLUSIONS

It appears from the results presented above that the high and invariant level of \(Mg^++\) concentration characteristic of the physiological state is crucial to the rate of energy transduction. At this level both maximum inhibition and maximum activation occur on formation and disruption of the postulated \(Mg^++\)-chelate complex. The striking conclusion emerges that this transition is brought about by the ability of actin to block one of the participating SH sites in the chelate. It seems possible that the tension-generating event may be directly related to a conformational change that is coupled to the opening of the \(Mg^++\)-chelate complex.

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