Calcium Sensitivity of Actomyosin ATPase: Its Modification by Substitution of Myosin Sulfhydryl Groups

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SH group substitution by DTNB enabled natural actomyosin to split ATP (in the presence of Mg^{2+}) also in the absence of Ca^{2+}, when assayed at low ionic strength. At higher KCl concentrations the ATPase activity of SH group substituted actomyosin was still Ca-dependent. Addition of unsubstituted myosin to natural actomyosin whose SH groups had been substituted increased the ATPase activity. This increase was Ca-insensitive indicating that SH group substitution of myosin in actomyosin can make the interaction of additional myosin molecules Ca-independent. In natural actomyosin Ca-insensitivity of ATPase activity was attained at a lower degree of SH group substitution when substitution was performed in the presence of EDTA. The part of ATPase activity which still remained Ca-sensitive after DTNB treatment could be activated by lower concentrations of free Ca^{2+} than the Ca-sensitive ATPase of untreated actomyosin. In reconstituted actomyosin the Ca-sensitivity of ATPase activity could more easily be reduced when the myosin-actin ratio was high. For demonstrating remaining Ca-sensitivity in SH group substituted reconstituted actomyosin more tropomyosin-troponin was needed than for sensitizing unsubstituted actomyosin to Ca^{2+}. — The similarities between the ATPase activity of SH group substituted actomyosin on the one hand and that of actomyosin at low concentrations of ATP on the other hand suggest that SH group substitution modifies actin-myosin interaction in a similar way as does nucleotide-free myosin (rigor myosin).

Introduction

It is known for some years that modification of sulfhydryl groups of actomyosin can abolish the Ca-sensitivity of actomyosin ATPase: After SH group substitution the ATPase is no longer inhibited by Ca-removal in the presence of the regulatory proteins tropomyosin and troponin [1–4]. We [4] and other groups too [3, 5, 6], presented evidence that the SH groups involved are located at the myosin molecule. However, at least in our hands, the modification of isolated myosin by SH group substitution does not suppress Ca-sensitivity. Instead, the entire actomyosin complex had to be incubated with the SH reagent. A more specific reactivity of actomyosin as compared to myosin is reported also by Daniel and Hartshorne [5].

The conclusion that myosin is the target of the SH reagent is based mainly on the following observation: After the incubation of actomyosin with the SH reagent myosin has been separated by ultracentrifugation in the presence of ATP and 0.6 M KCl from actin and then added to new regulated actin (actin that contains the regulatory proteins tropomyosin and troponin). This new actomyosin formed from modified myosin and regulated actin that had not been in contact with the SH reagent was Ca-insensitive [4, 5]. However, we sometimes found that the new actomyosin which contains myosin that was formerly modified as part of another actomyosin was none the less Ca-sensitive. We, therefore, reinvestigated this problem. The main result of our study is that substitution of myosin SH groups does not totally destroy Ca-sensitivity but rather alters the conditions under which Ca-sensitivity can be detected.

Parts of these results have been presented at the 9th International Congress of Biochemistry at Stockholm in July 1973 [7].

Abbreviations: DTNB, 5,5′-dithio-bis-(nitrobenzoic acid); EGTA, ethyleneglycol-bis-(aminoethyl)-N,N′-tetraacetic acid.

Enzymes: ATPase, ATP-phosphohydrolase, EC 3.6.1.3.

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Methods and Materials

The preparation of myosin, actin, natural actomyosin (the latter is prepared as entire actomyosin complex containing the regulatory proteins tropomyosin and troponin) and tropomyosin-troponin (prepared as the entire complex) has been de-

scribed elsewhere [8–10]. Reconstituted actomyosin refers to actomyosin that has been prepared by mixing separately prepared actin and myosin. Regulated actin refers to actin that contains the regulatory proteins tropomyosin and troponin.

The measurements of ATPase activity have been performed as described earlier [8, 9]. The constituents of the ATPase assays (10 ml) were normally as follows (unless otherwise stated): ATP and $MgCl_2$: 2 mM, KCl: 0.03 M, Tris-maleate buffer (pH 7.2): 20 mM, actomyosin: about 0.2 mg/ml. 100% ATPase activity of the single experiments refer to values in the range of $0.3-0.4$ μmol Pi $\times$ min^{-1} $\times$ mg actomyosin (or myosin).

Incubation of actomyosin with DTNB was normally performed in 0.6 M KCl at pH 8.0 (20 mM Tris-HCl buffer) at 22 °C for 20 min. The concentration of natural actomyosin in the incubation assays was about 3 mg/ml, that of the constituents of reconstituted actomyosin is indicated in the legends of figures and table. After incubation 0.5 ml of the incubation assay was transferred to the splitting assays and ATP hydrolysis was measured immediately after the incubation with DTNB. The extent of reaction between DTNB and proteins was measured after 20 min incubation in a Pye Unicam Spectrophotometer at 412 nm (in 0.6 M KCl and pH 8.0). The amount of substituted SH groups was calculated using a molar absorption coefficient for the coloured anion of 13600 [11].

In those cases in which no unreacted DTNB should be transferred into the splitting assay (in order to avoid substitution of proteins other than the desired ones), the incubation assay was diluted twentifold with water and the precipitated actomyosin was dissolved in 0.6 M KCl prior to the transference into the splitting assay. These cases are indicated in the legends of the figures.

Results

SH group substitution of natural actomyosin

Fig. 1 shows the already known fact [4] that addition of increasing amounts of DTNB to natural actomyosin (prepared from muscle as the entire actotropomyosin-troponin-myosin complex) not only activated actomyosin ATPase but reduced the Ca-sensitivity of this actomyosin ATPase. There is still another important result to be seen from Fig. 1: When SH group substitution of actomyosin was done in the presence of EDTA, Ca-sensitivity was suppressed at a much lower DTNB concentration than in the absence of EDTA. This result was not

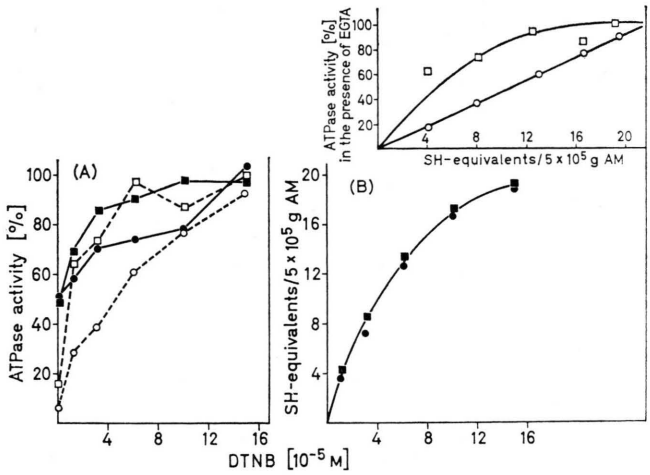

Fig. 1. ATPase activity and amount of substituted SH groups of natural actomyosin (AM) after incubation with DTNB. The incubation with DTNB was performed either in the absence (●, ○) or presence (■, □) of 1 mM EDTA. Open symbols represent ATPase activity in the presence of 1 mM EGTA. — A. ATPase activity. The ATPase assay contained 0.13 mg/ml actomyosin. — B. Amount of substituted SH groups. Inset: Relation between amount of substituted SH groups and ATPase activity in the presence of EGTA. The corresponding values have been taken from A and B, respectively. The curved line in the inset represents the function $A=100[1-(1-p)^3]$ where A is ATPase activity and p is the probability of substitution of those SH groups whose substitution makes ATPase activity possible also in the absence of Ca^{2+}. In this particular case p has been taken as proportional to the number of total SH groups substituted with $p=1$ when 21 SH groups are substituted. This relation would prevail if the substitution of one essential SH group would also modify the behaviour of two adjacent ones.

due to a higher affinity of DTNB to actomyosin in the presence of EDTA. Although under specific circumstances (especially at low ionic strength and when HMM rather than myosin is used) myosin SH groups may be more readily substituted in the presence of EDTA (compare also Schaub *et al* [12]), it can be seen from Fig. 1 B that in the case of the experiment of Fig. 1 the number of substituted SH groups was the same in the absence and presence of EDTA.

The inset of Fig. 1 relates the ATPase activity measured in the absence of Ca^{2+} to the amount of substituted sulfhydryl groups. As can be seen, when SH group substitution was performed in the absence of EDTA the ATPase activity which was afterwards measured in the absence of Ca^{2+} was proportional to the amount of SH groups substituted and attained its maximum when 21 SH equivalents per

500 000 g of actomyosin were substituted. This proportionality means that the SH groups the substitution of which enabled the ATPase to become active also in the absence of Ca^{2+} did not differ in their reactivity to DTNB from the total population of SH groups which could be substituted by DTNB. When SH group substitution was performed in the presence of EDTA the relation between the number of substituted SH groups and ATPase activity (measured in the absence of Ca^{2+}) was consistent with the assumption (compare the curved line in the inset) that in order to enable actomyosin to split ATP also in the absence of Ca^{2+} not every SH group that is involved in Ca-sensitivity has to be substituted. Instead, it can be deduced that the substitution of one SH group modifies also the behaviour of two adjacent ones (compare [13] for analogous statistical considerations concerning functional groups of enzymes).

Fig. 2 shows that the reduction of Ca-sensitivity was visible only under particular conditions. It can be seen that suppression of Ca-sensitivity of actomyosin ATPase was complete only when the ATPase was assayed at very low ionic strength. When measured at higher values of ionic strength the ATPase activity was Ca-sensitive in spite of SH group substitution.

This reappearance of Ca-sensitivity under splitting conditions of higher ionic strength is more explicitly shown in Fig. 3. SH group substitution shifted the range of ionic strength within which ATPase activity is possible to higher values of ionic strength (*cf.* [4]) whereas tropomyosin-troponin (in the absence of Ca^{2+}) shifted this range to lower values of ionic strength. Hence, in the experiment of Fig. 3 the relation between actomyosin ATPase activity (in the absence of Ca^{2+}) and ionic strength was shifted to such an extent to higher values of ionic strength that in 0.03 M KCl the ATPase activity was similar both in the absence and presence of Ca^{2+}. But still the decline of ATPase activity began to occur at a lower value of ionic strength when Ca^{2+} was absent than when it was present. When actomyosin, however,was incubated with DTNB in the presence of EDTA (Fig. 3 B) the effect of substitution was so pronounced that now the ionic strength dependence of ATPase activity was equal in the absence and presence of Ca^{2+} so that Ca-sensitivity did not reappear.

That ionic strength influences the extent of Ca-sensitivity of actomyosin systems appears to be a general phenomenon not restricted to SH group substituted actomyosin. Thus, myofibrillar ATPase

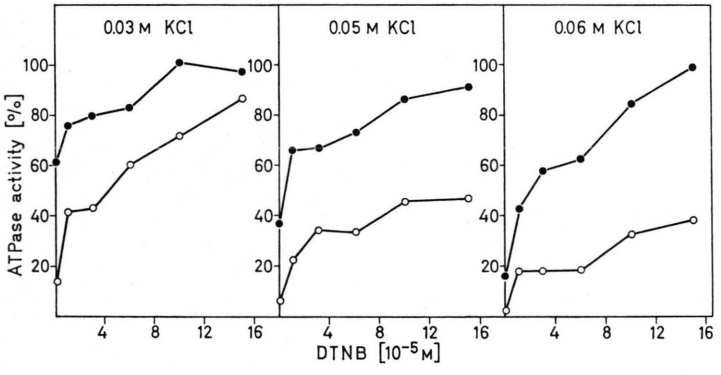

Fig. 2. ATPase activity of natural actomyosin after incubation (in 0.6 M KCl) with the DTNB concentrations indicated at the abscissa. ATPase activity was measured at the concentrations of KCl indicated in the figure. The open symbols refer to ATPase activity in the presence of 1 mM EGTA. The actomyosin concentration of the splitting assays was 0.11 mg/ml.

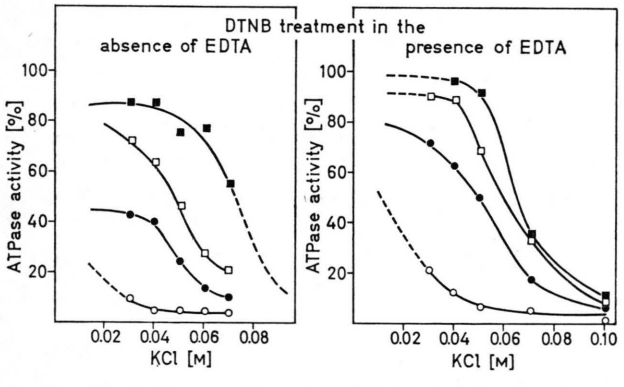

Fig. 3. ATPase activity of DTNB incubated natural actomyosin as dependent on the ionic strength of the ATPase assay (KCl concentrations indicated at the abscissa). Natural actomyosin was incubated with DTNB either in the absence or presence of 1 mM EDTA in 0.6 M KCl and 0.1 mM DTNB. ●, ○ control (the incubation medium did not contain DTNB); ■, □ DTNB treated actomyosin. Open symbols represent ATPase activity in the presence of 1 mM EGTA.

Table. Relative ATPase activity (%) of natural actomyosin after the addition of myosin or tropomyosin-troponin. Natural actomyosin has been incubated in 60 μM DTNB in the presence of 1 mM EDTA or has been kept as control without DTNB. After DTNB incubation the actomyosin has been diluted twentifold, precipitated and redissolved in 0.6 M KCl to remove unreacted DTNB. ATPase activity has been measured either in the absence or presence of 1 mM EGTA in 10 ml assays containing 0.55 mg actomyosin and (if added) the following additional proteins which have not been in contact with DTNB: 1.2 mg myosin or 0.9 mg tropomyosin-troponin. 100% ATPase activity was 0.8 μmol Pi × mg actomyosin^{-1} × min^{-1}.

Actomyosin				Actomyosin plus tropomyosin-troponin				Actomyosin plus myosin			
−DTNB		+DTNB		−DTNB		+DTNB		−DTNB		+DTNB	
− EGTA	+ EGTA	− EGTA	+ EGTA	− EGTA	+ EGTA	− EGTA	+ EGTA	− EGTA	+ EGTA	− EGTA	+ EGTA
35	9	75	65	39	7	85	45	88	22	99	94

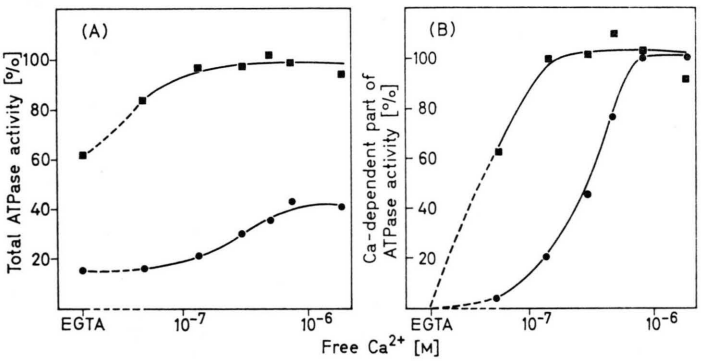

Fig. 4. Dependence of ATPase activity of natural actomyosin on concentration of free Ca^{2+}. Calcium concentration has been adjusted with CaEGTA and EGTA (5 mM each) using a stability constant for CaEGTA of 5×10^6 M^{-1}. For SH group substitution natural actomyosin was incubated for 20 min with 0.1 mM DTNB. In A the total ATPase activity is shown. In B only that part of the ATPase activity is shown that changed with Ca^{2+} concentration. At each Ca^{2+} concentration the difference between the ATPase activity at that particular Ca^{2+} concentration and the ATPase activity at 1 mM EGTA was plotted in % of the difference between the ATPase activity at 2×10^{-6} M Ca^{2+} and that at 1 mM EGTA. ● control, ■ DTNB-incubated actomyosin.

requires less Ca^{2+} at low ionic strength than at higher ionic strength [14] and resting tension (tension in the presence of EGTA) of skinned muscle fibers increases as the ionic strength decreases [15, 16].

The Table reveals that not only after the elevation of ionic strength Ca-sensitivity reappeared. Natural actomyosin (which contains tropomyosin-troponin by itself) which has become Ca-insensitive after SH group substitution regained a considerable Ca-sensitivity after addition of tropomyosin-troponin. Still another surprising result is demonstrated in the table: After the addition of myosin to substituted actomyosin the ATPase activity rose above the value of that of actomyosin alone, but this increment, which reflects the ATPase activity of the newly added myosin, was none the less Ca-insensitive although this added myosin had not been in contact with the SH reagent.

Bremel and Weber [17] have shown that at low concentrations of ATP, when Ca-sensitivity of actomyosin is suppressed [18], myofibrils bind Ca^{2+} with a higher affinity than at high concentrations of ATP. From Fig. 4 it can be seen that the part of ATPase activity that remains still Ca-sensitive after SH group substitution can be activated by lower Ca^{2+} concentrations than the ATPase of unsubstituted actomyosin. This may be taken as first evidence that SH group substituted myosin and "rigor myosin" (myosin free of nucleotides) may suppress Ca-sensitivity through common mechanisms.

SH group substitution of reconstituted actomyosin

The conditions which lead to a suppression of Ca-sensitivity by SH group substitution have to be rather specific. We never succeeded in reducing Ca-sensitivity when we incubated only the myosin component [4]. The presence of actin was in our hands a necessary prerequisit for the reduction of Ca-sensitivity. There is no straightforward explanation why only "rigor links" can be transformed

into Ca-insensitive myosin. But even then specific conditions have to be fulfilled, so that not in every experiment there was a reduction of Ca-sensitivity of reconstituted actomyosin. Reduction of ionic strength during incubation with DTNB did not improve the conditions for the reduction of Ca-sensitivity (contrary to the experience of Daniel and Hartshorne [5]), although this enhanced the activation by DTNB of the ATPase activity of actomyosin. In reconstituted actomyosin EDTA (in contrast to natural actomyosin) did not facilitate the suppression of Ca-sensitivity.

The conditions which affect the reactivity of reconstituted actomyosin are exemplified by the experiments to be described in Figs 5 and 6.

Fig. 5 shows an experiment in which different amounts of tropomyosin-troponin were added to reconstituted actomyosin. It can be seen that in the unsubstituted control actomyosin ATPase activity (in the absence of Ca^{2+}) was strongly inhibited by the addition of tropomyosin-troponin. In substituted actomyosin the ATPase activity (in the absence of EGTA) was markedly higher than in unsubstituted actomyosin. In the presence of such amounts of tropomyosin-troponin which sensitized the unsubstituted actomyosin to Ca^{2+} only a minor inhibition by EGTA could be seen. With increasing amounts of tropomyosin-troponin, however, Ca-sensitivity increased, although it did not reach the high degree of sensitivity that could be observed in unsubstituted actomyosin.

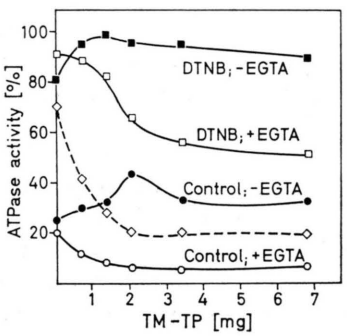

Fig. 5. ATPase activity of reconstituted actomyosin after addition of varying amounts of tropomyosin-troponin. The DTNB treated actomyosin (19 mg actin and 20 mg myosin in 13 ml containing 0.1 mM DTNB) was precipitaded (after twentifold dilution with H_2O) and redissolved in 7 ml 0.6 M KCl in order to remove unreacted DTNB before transference to the splitting assay. The splitting assay (10 ml) contained besides the DTNB reacted actomyosin (0.5 ml) the amounts of tropomyosin-troponin (TM-TP) indicated at the abscissa. ●, ○ incubation medium without DTNB; ■, □ incubation medium containing 0.1 mM DTNB. Open symbols represent ATPase activity in the presence of 1 mM EGTA. The interrupted curve has been obtained by enlarging the EGTA curve of the control to such an extent that its relative course can be better compared with the EGTA curve of the DTNB treated actomyosin.

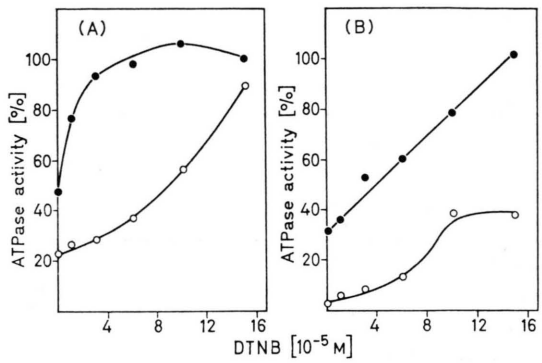

Fig. 6. Different effects of DTNB treatment on reconstituted actomyosin which differed in the actin-myosin ratio. The DTNB treatment was performed in assays (1.33 ml) containing 2.15 mg actin and the following amounts of myosin: 3.0 mg in (A) or 0.7 mg in (B) and the DTNB concentrations indicated at the abscissa. The open symbols represent ATPase activity in the presence of 1 mM EGTA.

When the "EGTA-curve" of unsubstituted actomyosin is enlarged to such an extent that the relative course of both EGTA curves can be compared (Fig. 5) it is obvious that the apparent affinity of tropomyosin-troponin is remarkably higher to unsubstituted than to substituted actomyosin.

The role of actin-myosin ratio on the effect of SH group substitution

The experience that addition of untreated myosin to SH group substituted actomyosin restored (at least partially) Ca-sensitivtiy as well as the well-known fact (see Weber and Murray [18] for review) that Ca-sensitivity can also be suppressed by lowering the ATP concentration led to the supposition that the interaction between SH group substituted myosin and actin has some features in common with the interaction between nucleotide-free myosin (rigor myosin) and actin (compare references [17, 19, 10]). If so, one should expect that substituted myosin acts the better the more densely it is populated on the actin filament. Fig. 6 B shows that the ATPase activity of actomyosin with a low content of myosin could be strongly activated by DTNB, but its Ca-

sensitivity did not disappear. However, when the same amount of actin was combined with a large excess of myosin (Fig. 6 A) Ca-sensitivity was nearly completely suppressed by DTNB.

A further difference between actomyosin with different myosin content can be seen: Whereas in actomyosin with the low myosin content the activation of ATPase activity by DTNB (measured in the absence of EGTA) did not reach a plateau, it did reach a maximal value in the actomyosin with the high myosin content. Presumably, the more densely myosin is packed on the thin filament the fewer myosin molecules have to be substituted in order for actomyosin ATPase to become enhanced and to become Ca-insensitive. For abolishing Ca-sensitivity there seems to exist a minimum critical density on actin which has not been reached in the experiment of Fig. 6 B.

Discussion

The model

The present results have shown that the enzymatic interaction between actin and myosin whose SH groups were substituted in the presence of actin has many features in common with the interaction between actin and myosin in the presence of low ATP concentrations, when rigor links exist. In both cases the Ca-sensitivity of actomyosin ATPase is reduced, the apparent affinity of Ca^{2+} to the contractile proteins is increased and the actin-myosin ratio plays an important role. This suggests that the results of this paper can be explained with the aid of a model whose structural parts were proposed by Parry and Squire [20], Haselgrove [21] and Huxley [22]. It is assumed that the two tropomyosin strands which are arranged parallel to the actin superhelix can move on the actin filament between two extreme positions, one nearer to the groove of the actin filament and the other nearer to the periphery of the actin filament. When tropomyosin is near the groove of the actin filament the whole thin filament is able to activate myosin ATPase even more efficiently than unregulated actin can do (high-affinity state of Dancker [10]). When being in a position near the periphery of the actin filament tropomyosin blocks the interaction between actin and myosin (low affinity state of Dancker [10]). The present experiments suggest that the reduction of Ca-sensitivity after the substitution of myosin SH groups can be explained by assuming that modified myosin has the ability to shift (during the enzymatic interaction with actin) tropomyosin in the direction of the groove of the thin filament, thus counteracting the opposite effect which is exerted on tropomyosin-troponin by Ca-removal. In this way the Ca-insensitivity of actomyosin ATPase in the presence of rigor links has been explained [17–19]. The actual position which is adopted by tropomyosin is supposed to depend on the ratio of all influences which tend to shift tropomyosin in the one or other direction. Hence, if all influences are absent or balance each other, tropomyosin should lie somewhere between the extreme positions (intermediate state of Dancker [10]).

This model further explains that when myosin is densely populated on the actin filament, a smaller proportion of the total myosin needs to be substituted by DTNB in order to reach the critical concentration of substituted myosin on actin which is necessary to shift tropomyosin into the groove than when myosin is widely populated. In the latter case the critical density may not be reached even when the whole myosin population is substituted (Fig. 6 B). On the other hand, if the critical density has been reached, additional unsubstituted myosin can interact with actin only in a Ca-insensitive manner (Table) .

That Ca-sensitivity can be more easily seen at higher ionic strength (Figs 2 and 3) can be explained in the following way: Since elevation of the ionic strength of the ATPase assay reduces the apparent affinity of actin to myosin [23, 24], one can expect that at higher ionic strength there is less substituted myosin in contact with the thin filament than is needed to resist the movement of tropomyosin (induced by the removal of Ca^{2+}) from the central position in the groove to the peripheral position.

The problem of the varying amounts of tropomyosin-troponin needed for Ca-sensitivity

Difficult to explain is why higher amounts of tropomyosin-troponin are able to restore Ca-sensitivity of SH group substituted actomyosin. From the experiments of Fig. 6 one may infer that more tropomyosin-troponin is needed to saturate the binding sites for tropomyosin-troponin of SH group substituted actomyosin. Then one should assume that SH group substitution of natural acto-

myosin (compare Table) dissociates the endogenous tropomyosin-troponin from actomyosin and that the empty sites can be refilled by sufficiently high amounts of added tropomyosin-troponin. Alternativly there may be less tropomyosin-troponin needed to relax unsubstituted than substituted actomyosin. This would imply, however, that the ratio of actin to tropomyosin-troponin which is widely believed to be a constant one [19, 25] may vary.

The number of SH group involved in Ca-sensitivity

The results of Fig. 1 propose that, when SH group substitution has been performed in the presence of EDTA, the substitution of one SH group modifies also the behaviour of two more SH groups. In view of the results discussed above (see Table) that not all myosin molecules need to be substituted in order to make the interaction of the whole population of myosin molecules with actin Ca-insensitive (indicating a "cooperation" between different myosin molecules), it seems resonable to assume that the three SH groups whose behaviour is governed by the substitution of only one SH group reside in three different myosin molecules (or heads). This suggests that only one SH group per active site is involved in making the interaction between myosin and regulated actin Ca-sensitive. Daniel and Hartshorne [5] have reported that 3 to 5 molecules of SH-reagents were incorporated into one myosin molecule when Ca-sensitivity of actomyosin has disappeared, but this number can only be an upper limit.

In conclusion: The enzymatic interaction between myosin and regulated actin depends not only on Ca^{2+} concentration but also on the absence or presence of a specific influence which can be exerted by myosin on the thin filament. This influence cannot only be exerted by rigor links [17–19] but also by myosin molecules the SH groups of which are substituted under specific conditions. Accordingly, Ca-sensitivity of actomyosin ATPase depends not only on the state of the thin filament but also on the state of myosin.

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