Interactions of Desensitized Actomyosin with Tropomyosin, Troponin A, Troponin B, and Polyanions

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ABSTRACT Troponin B is an inhibitor of the Mg\(^{2+}\)-activated ATPase activity of actomyosin. The inhibitory effect, which is observed, however, depends upon whether tropomyosin is also present. In the absence of tropomyosin the inhibition by troponin B is markedly reduced by increasing the ionic strength from 0.03 to 0.07, but is not affected by calcium up to a concentration of \(10^{-4}\) M. Troponin A relieves the inhibition in both the absence and presence of calcium, an effect which is also shown by many polyanions and is illustrated by using RNA. Tropomyosin enhances the inhibitory effect of troponin B and renders it more resistant to increasing ionic strength but it does not make the inhibition calcium-sensitive. However, when troponin A or low concentrations of polyanions are added to troponin B and tropomyosin, the actomyosin ATPase activity becomes calcium-sensitive; i.e., in the presence of tropomyosin, troponin A or polyanions do not relieve the inhibitory action of troponin B in the absence of calcium but only in its presence. In marked contrast to this is the effect of troponin A in the absence of tropomyosin where it neutralizes the effect of troponin B under all conditions. Thus troponin A and the polyanions both confer calcium regulation on the troponin B–tropomyosin system. The similar effects exhibited by troponin A and the polyanions suggest that the addition of net negative charge to troponin B is an important factor in the conferral of calcium sensitivity. It is also clear that tropomyosin is an essential component of the regulatory mechanism.

INTRODUCTION

The regulation of myofibrillar ATPase activity by calcium is a complex mechanism and involves several factors. Ebashi (1963) was the first to recognize that a protein component other than actin and myosin was necessary and subsequently Ebashi and Kodama (1965) found that this component was not

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actually two proteins. One of them proved to be tropomyosin (Bailey, 1948) while the other was a previously unrecognized protein which was later termed troponin (Ebashi and Kodama, 1966 a). Both troponin and tropomyosin are essential for the calcium-dependent relaxation of the actomyosin system (Ebashi and Kodama, 1966 b; Hartshorne and Mueller, 1967).

More recently, troponin has been separated into two functional components, troponin A and troponin B (Hartshorne and Mueller, 1968). Troponin B was found to be an inhibitor of actomyosin ATPase activity whose effect was not influenced by the presence of low concentrations of calcium. The addition of tropomyosin to troponin B enhanced the inhibition, which remained essentially calcium-insensitive. Tropomyosin and troponin B interacted to form a discrete sedimenting boundary and both the complex formation and inhibition of ATPase activity appeared to be optimal at an equal weight ratio of the two proteins (Hartshorne et al., 1969).

Troponin A by itself had little effect on the ATPase activity of actomyosin. However, if troponin A was added to a system containing actomyosin, troponin B, and tropomyosin a marked effect was observed only in the presence of calcium: the inhibition of the actomyosin ATPase activity by the complex of troponin B and tropomyosin was removed. Since this inhibition was not removed in the absence of calcium, it appeared that troponin A conferred calcium regulation on the inhibitory action of troponin B and tropomyosin.

From amino acid analyses reported earlier (Hartshorne and Mueller, 1968) it was noticed that troponin A at neutral pH, possesses a higher net negative charge than troponin B. It was also observed (Hartshorne et al., 1969) that two polyanions, heparin and polyglutamic acid, were capable of producing some of the effects of troponin A. In order to determine whether the action of troponin A was dependent on its net negative charge, a more detailed study of the interaction of troponin B with troponin A and various polyanions was undertaken. This, together with additional data on the troponin system, is presented here.

**EXPERIMENTAL**

Troponin and tropomyosin were prepared from the P₄₀₋₄₅ fraction as described previously (Hartshorne and Mueller, 1969), with the following minor modification for troponin. The supernatant which was obtained at pH 4.6 from the P₄₀₋₄₅ fraction was neutralized, dialyzed against 4 volumes of 1 mM dithiothreitol, 10 mM Tris-HCl (pH 7.6), and adjusted to 60% ammonium sulfate saturation. The resulting precipitate which consisted of troponin was dissolved and dialyzed against 1 mM KCl, 1 mM dithiothreitol. This procedure reduced contamination by RNA and was also more convenient than the previously reported ultrafiltration method.

1 Previously, an "inhibitory factor" was described by Hartshorne et al. (1967). In the light of more recent results, this appears to be essentially an impure preparation of troponin B.
The separation of tropomycin into the A and B components was carried out as described previously (Hartshorne and Mueller, 1968).

Enzymic Assays The basic medium for the assay of the Mg$^{++}$-activated ATPase activity of actomyosin was 2.5 mM MgC$_2$, 2.5 mM ATP, and 25 mM Tris-HCl (pH 7.6). EGTA (1 mM) was included in this medium when Ca$^{++}$-free conditions were desired. Assays were performed in both the basic medium and that containing the EGTA to assess the calcium sensitivity of a particular system. The calcium content of the Mg$^{++}$ assay medium (in the absence of EGTA) and the calcium introduced with the proteins were adequate to saturate tropomycin. Usually in this medium a final concentration of approximately 10$^{-4}$ M calcium was obtained. When known low calcium concentrations were required, the calcium buffer system of CaEGTA and EGTA was used, assuming an apparent dissociation constant at pH 7.6 of 1.2 × 10$^{-8}$.

Assays were carried out at 25°C in a final volume of 2 ml and the reaction was terminated by the addition of 1 ml of 25% trichloroacetic acid. Desensitized actomyosin, prepared as described by Schaub et al. (1967), was used for all these studies. Other assay conditions will be indicated in the text.

Protein Concentrations These were estimated from microKjeldahl nitrogen analyses assuming a nitrogen content of 16%.

Reagents The polyanions which were used were: heparin, sodium salt (Fisher Scientific Co., and Sigma Chemical Co.); polyglutamic acid, sodium salt (Pilot Chemicals, Inc.); polyanethol sodium sulfonate (Fisher Scientific Co.); polyadenylic acid, polyuridylic acid, and polycytidylic acid (Miles Laboratories, Inc.); RNA and DNA (Calbiochem). ATP, disodium salt, was obtained from Sigma Chemical Co. All other chemicals were reagent grade.

RESULTS

Fig. 1 shows that the inhibition of actomyosin ATPase activity by troponin B was not removed when the calcium concentration was raised from values of approximately 10$^{-8}$ to 10$^{-4}$ M. There was no effect of calcium at all when troponin B alone was used. When troponin B was present with tropomyosin (Fig. 2), calcium in the range from 0.2 to 10 μM caused a slight activation, increasing the ATPase activity from 46 to 54% of that in the absence of troponin B. At the moment it is not possible to decide whether this effect was due to slight contamination of troponin B by troponin A, although repeated fractionation of troponin B at pH 1 did not alter the situation. Thus it must also be considered that a more efficient inhibition at very low concentrations of calcium may be an inherent feature of the troponin B–tropomyosin complex. However, it is apparent that the effect of calcium was very small and the inhibition by troponin B with or without tropomyosin may be regarded essentially as calcium-insensitive.

In Fig. 3 it is shown that increasing the ionic strength considerably decreased the inhibition by troponin B alone. At an ionic strength of 0.03 the
Figure 1. Effect of Ca\(^{++}\) concentration on the inhibition by troponin B of the Mg\(^{++}\)-activated ATPase activity of desensitized actomyosin. Troponin B constant (200 \(\mu\)g) for each point. Ca\(^{++}\) concentrations below approximately \(3.5 \times 10^{-6}\) M (pCa = 5.46) were obtained using different ratios of CaEGTA and EGTA, higher values were obtained by the addition of CaCl\(_2\). Actomyosin 0.25 mg/ml. The control ATPase activity of actomyosin in the basic Mg\(^{++}\) medium, in the absence of troponin B, was 61 \(\mu\)g P\(_1\) liberated/5 min per mg actomyosin.

ATPase activity was reduced to approximately 30\% by 270 \(\mu\)g of troponin B, whereas at an ionic strength of 0.07 the same amount of troponin B reduced the activity to only approximately 60\%.\(^2\) Frequently small amounts of troponin B (or a contaminant contained in the preparation) caused a slight acti-

\(^2\) It should be mentioned that the slight inhibitory effect of unfractionated troponin (Hartshorne and Mueller, 1968) was found to be similarly sensitive to changes in ionic strength. In the presence of 40 mM KCl, no inhibition was observed with up to 400 \(\mu\)g of troponin in either the Mg\(^{++}\) or Mg\(^{++}\) EGTA assay media. It is clear from this that any comparative studies made on troponin or troponin B must be performed under carefully defined ionic conditions.
vation of ATPase activity at the higher values of ionic strength (Fig. 3). The drop in the percentage of inhibition primarily reflects the decrease of the control ATPase activity: high concentrations of troponin B (greater than 200 µg/assay) reduced the ATPase activity to the same low rate at all values of ionic strength. The inhibitory effect of different samples of troponin B was

![Graph showing the effect of increasing ionic strength on the inhibition by troponin B of the Mg²⁺-activated ATPase activity of desensitized actomyosin. Assays performed in the basic Mg²⁺ medium containing: 0 mm KCl [(□) I \(\approx 0.03\)], 10 mm KCl [(●) I \(\approx 0.04\)], 20 mm KCl [(□) I \(\approx 0.05\)], 30 mm KCl [(●) I \(\approx 0.06\)], 40 mm KCl [(△) I \(\approx 0.07\)]. Actomyosin 0.30 mg/ml.](image)

variable, and the amounts of troponin B required for 50% inhibition of ATPase activity (at I \(\approx 0.03\)) ranged from 90 to 200 µg, with about 130 µg for the majority of preparations.

Although increasing salt concentrations greatly reduced the rate of ATP hydrolysis, the inhibitory effect of troponin B was not decreased if tropomyosin was also present. In the presence of calcium the reduction of the ATPase activity to about 35% remained unaltered when the ionic strength was increased from 0.03 to 0.07 (Fig. 4 A). In the absence of calcium at the lowest ionic strength, the ATPase activity was reduced to about 25%. An increase in the
FIGURE 4. Effect of increasing ionic strength on the inhibition by the troponin B–tropomyosin complex of the Mg$^{++}$-activated ATPase activity of desensitized actomyosin. A, assays performed in basic Mg$^{++}$ medium. B, assays performed in Mg$^{++}$-EGTA medium (see Experimental). Additions, 0 mM KCl [(O)] I = 0.03, 10 mM KCl [(●)] I = 0.04, 20 mM KCl [(□)] I = 0.05, 30 mM KCl [(■)] I = 0.06, 40 mM KCl [(△)] I = 0.07. Troponin B and tropomyosin combined in a 1:1 weight ratio. Actomyosin 0.24 mg/ml.

FIGURE 5. Effect of troponin A on the inhibition by the troponin B–tropomyosin complex of the Mg$^{++}$-activated ATPase activity of desensitized actomyosin. Troponin B and tropomyosin combined in a 1:1 weight ratio, 100 µg mixture used for each point. Assays performed in basic Mg$^{++}$ medium (O), and in Mg$^{++}$-EGTA medium (●) (see Experimental). Dashed line indicates level of control ATPase activity. Actomyosin 0.26 mg/ml.
ionic strength actually slightly enhanced the inhibition, and at $I \approx 0.07$ the ATPase activity was inhibited to about 18% of the control level (Fig. 4 B).

In Fig. 5 it is demonstrated that calcium sensitivity is introduced into the troponin B—tropomyosin complex by the addition of troponin A. In the absence of calcium the inhibition persisted after the addition of troponin A; whereas in the presence of calcium (i.e., in the Mg$^{++}$ assay medium where the calcium introduced with the proteins, ATP, etc. yielded a final Ca$^{++}$ concen-

![Figure 6](image_url)

**Figure 6.** Effect of increasing ionic strength on the inhibition by the troponin-tropomyosin complex of the Mg$^{++}$-activated ATPase activity of desensitized actomyosin. A, assays performed in basic Mg$^{++}$ medium. B, assays performed in Mg$^{++}$ EGTA medium (see Experimental). Additions, 0 mm KCl [○] $I \approx 0.03$, 10 mm KCl [●] $I \approx 0.04$, 20 mm KCl [□] $I \approx 0.05$, 30 mm KCl [■] $I \approx 0.06$, 40 mm KCl [Δ] $I \approx 0.07$. Troponin and tropomyosin combined in a 1.3:1 weight ratio, respectively. Actomyosin 0.24 mg/ml.

tration approaching $10^{-4} M$) the inhibition was removed and a slight activation above the control ATPase level was observed on the addition of more than approximately 15 µg of troponin A. The activation effect was found over a range of ionic strengths. This is shown by the experiments illustrated in Fig. 6 A, for which the separate addition of troponin A and B was replaced by the addition of unfractonated troponin. In the absence of calcium the inhibition by troponin and tropomyosin persisted over the indicated range of ionic strengths (Fig. 6 B) and was similar to that observed with troponin B and tropomyosin. A comparison of Figs. 4–6 indicates that the isolation procedures used for troponin A and B did not significantly alter these components relative to their state in the parent troponin.
As previously reported, the amino acid analyses of troponin A and troponin B (Hartshorne and Mueller, 1968) indicate that troponin A possesses a higher net negative charge than troponin B, because troponin A contains considerably less arginine and lysine than B. A possible significance of the excess negative charge for the functioning of troponin A was suggested by the observation that heparin and polyglutamic acid were capable of producing some of the effects of troponin A (Hartshorne et al., 1969).

FIGURE 7. Effect on the Mg$^{++}$-activated ATPase activity of desensitized actomyosin of the addition of RNA or troponin A to tropomyosin. Solid symbols represent assays performed in basic Mg$^{++}$ medium and open symbols those performed in Mg$^{++}$ EGTA medium (see Experimental). Troponin A (■, ○), RNA (■, □). Tropomyosin constant (100 µg) for each point. Actomyosin 0.26 mg/ml.

Several polyanions have now been studied. It was found that all of them removed the inhibition of ATPase activity by troponin B. The polyanions were heparin, polyglutamic acid, polyanethol sodium sulfonate, polyadenylic acid, polyuridylic acid, RNA, and DNA. Some of these compounds themselves exerted a marked inhibitory effect on the Mg$^{++}$-activated ATPase activity of actomyosin, as Bárány and Bárány (1960) and Bárány and Jaisle (1960) have shown. For the purpose of making a clear comparison between troponin A and polyanions, a polyanion was chosen which did not by itself markedly affect the actomyosin ATPase activity. This was RNA.
As shown in Fig. 7, RNA added to a constant amount of tropomyosin caused a slight inhibition of ATPase activity, which was not significantly affected by the absence or presence of calcium. The observed inhibition was due mainly to the slight inhibitory action of RNA alone. For comparison, an experiment using troponin A instead of RNA is also shown in Fig. 7. Similarly some inhibition was found but the effect again was slight. The better inhibition in the Mg\(^{++}\) EGTA medium was probably due to a small amount of contamination of troponin A by troponin B.

![Figure 8](image)

**Figure 8.** Effect of RNA on the inhibition by troponin B of the Mg\(^{++}\)-activated ATPase activity of desensitized actomyosin. Assays performed in basic Mg\(^{++}\) medium (O) and in Mg\(^{++}\) EGTA medium (●) (see Experimental). Troponin B constant (160 μg) for each point. Actomyosin 0.27 mg/ml.

When RNA in increasing amounts was added to actomyosin inhibited by troponin B alone (no tropomyosin present), the inhibition was gradually removed in the absence as well as in the presence of calcium (Fig. 8). At a weight ratio of approximately 1:5, RNA to troponin B, respectively, little inhibition was apparent in the presence of calcium. Although the ATPase activity was slightly higher in the presence of calcium, the difference under the two assay conditions was small. By contrast RNA conferred a significant degree of calcium regulation on the inhibition by troponin B if tropomyosin was also present (Fig. 9 A). When RNA in amounts up to 10 μg was added to actomyo-
FIGURE 9. A, effect of RNA on the inhibition by the troponin B-tropomyosin complex of the Mg++-activated ATPase activity of desensitized actomyosin. Assays performed in basic Mg++ medium (O), and in Mg++ EGTA medium (●) (see Experimental). Troponin B and tropomyosin combined in a 1:1 weight ratio, 100 µg mixture used for each point. Dashed line indicates level of control ATPase activity. B, difference (ΔP̄) between points obtained in Mg++ and Mg++EGTA assay media. Actomyosin 0.24 mg/ml.

FIGURE 10. A, effect of heparin on the inhibition by the troponin B-tropomyosin complex of the Mg++-activated ATPase activity of desensitized actomyosin. The rest of the legend as in Fig. 9.
sin containing both troponin B and tropomyosin (50 µg of each), the ATPase activity was fully restored in the presence of calcium, but remained inhibited in its absence. On increasing RNA further the calcium sensitivity decreased. The different responses in the presence and absence of calcium can be more clearly illustrated by taking the difference between the ATPase activities in the Mg++ and Mg++ EGTA media at each point (ΔP,) and plotting this vs. RNA added (Fig. 9 B).

Similar results were obtained when heparin was used instead of RNA (Fig. 10), although heparin was effective at lower concentrations. The situation with heparin, however, was complicated by the fact that heparin itself is an effective inhibitor of ATPase activity (Bárány and Bárány, 1960).

**DISCUSSION**

The results presented above support the earlier contention (Hartshorne and Mueller, 1968) that troponin is composed of two functional components. Recent analyses of troponin A and troponin B have shown that neither is homogeneous and as yet the nature and extent of the contaminating proteins have not been elucidated. However, the presence of tropomyosin in either can be eliminated. Earlier observations (Hartshorne et al., 1969) indicated that the effectiveness of troponin A increased as the ratio of \( A_{278} : A_{260} \) decreased. This was subsequently traced to the presence of RNA and to a lesser extent DNA, both of which have effects similar to that of troponin A. The troponin A preparations which were used for the studies reported here contained considerably less nucleic acid, and phosphorus analyses indicated less than 1.5 moles of phosphorus/10⁵ g troponin A. As shown in Fig. 5, approximately 20 µg of troponin A were adequate for calcium regulation. The nucleic acid content of this amount of protein would be about 0.1 µg, which is clearly not enough to account for the regulatory effect (see Fig. 9 A). It is therefore unlikely that nucleic acid is a functional component of troponin “in situ.” Recently Drabikowski et al. (1969) have also shown this. To the present time there is no evidence to indicate that a nonprotein component is involved in the functioning of either troponin A or troponin B.

Troponin B is an inhibitor of the Mg++-activated ATPase activity of actomyosin regardless of the Ca++ concentration. In order to explain this effect it is logical to suppose the binding of troponin B to either actin or myosin. There is no direct evidence to favor either possibility, although binding to actin seems the most likely because then it is not necessary to invoke two separate mechanisms for troponin B, one in the absence of tropomyosin and another in its presence. Tropomyosin is known to bind to actin (Martonosi, 1962; Laki et al., 1962; Drabikowski and Gergely, 1962) and troponin B and intact troponin are bound to tropomyosin. This was shown for troponin by Ebashi and Kodama (1965) and later confirmed by Hartshorne and Mueller (1967).
who also demonstrated a complex formation between troponin B and tropomyosin (Hartshorne et al., 1969). Therefore, since in the presence of tropomyosin, troponin B is presumably bound to actin, it is convenient to assume that it may also be bound to actin when tropomyosin is absent, especially since its inhibitory effect on the ATPase activity is similar under both conditions. Other very circumstantial evidence in favor of troponin B binding to actin is that the Ca++-activated ATPase activity of myosin is not affected by troponin B. Also, Kominz and Maruyama (1967) have found that the amount of native tropomyosin (troponin plus tropomyosin) bound to myosin is very small.

The suggestion that troponin B can be bound to actin directly does not require one to postulate different binding properties for troponin B and intact troponin. Experiments apparently contradictory to this from Ebashi's laboratory showing that troponin does not bind to actin (Ebashi and Kodama, 1966 a; Endo et al., 1966) were done at an elevated ionic strength that largely abolished the effect of troponin B, also quite possibly by preventing binding. At the low ionic strength required to observe the inhibitory effect of troponin B intact troponin also showed some inhibition which disappeared on raising the ionic strength. (This demonstrates that the slight effect of troponin alone was not due to tropomyosin contamination since if it were, the inhibition would have been resistant to increasing ionic strength, see Fig. 6.) Thus the inhibitory action of troponin B, and to a lesser extent of troponin, may be explained by the assumption of binding to actin. Possibly the effect of increasing the ionic strength is to reduce the extent of this binding.

Tropomyosin protects the inhibitory effect of troponin B against increasing ionic strength presumably because troponin B remains bound to tropomyosin. It also, at all ionic strengths, reduces the amount of troponin B required for a given inhibition to about one-third of that required in its absence. This again may be due to the higher affinity of troponin B for tropomyosin as compared to actin, but may also indicate that troponin B can regulate more than 1 actin molecule in the presence of tropomyosin. It is known that in the intact myofibril, in the absence of calcium, 1 troponin protects several actins from interaction with myosin. Inhibition of the myofibrillar ATPase activity is accomplished even though there is only 1 troponin for about 7 actins (Ebashi et al., 1968).

Let us now consider the function of troponin A. By itself or with tropomyosin it had little effect on the actomyosin ATPase activity. With troponin B its effect was significantly different in the absence and presence of tropomyosin. In the absence of tropomyosin, troponin A neutralized the inhibition by troponin B, an effect that was also produced by several polyanions. This suggests that the function of troponin A is dependent on its net negative charge. In view of the similarity of the effects produced by troponin A and the polyanions we may assume that they act at the same site, and since troponin A and
the polyanions solubilize troponin B at low ionic strength, an interaction with troponin B is indicated. The reason why this complex is now no longer an effective inhibitor is not clear, and basically there are two possibilities to consider. Either its binding to actin is reduced or it remains bound to actin but is altered to a relatively ineffective form. It should be emphasized that in the absence of tropomyosin the neutralization of the troponin B inhibition by both troponin A and low concentrations of polyanions occurs in the absence and presence of calcium.

Bárány and Bárány (1960) have demonstrated that the polyanion, polyethensulfonate, is bound by myosin. Furthermore the persistence of RNA in myosin preparations (Mihalyi et al., 1957; Perry, 1960; Baril et al., 1964, 1966) may also indicate an interaction with myosin. Thus the polyanion results are complicated by the possibility that there are two sites for polyanion interaction, troponin B and myosin. If heparin or polyglutamic acid is added to actomyosin, the ATPase activity is inhibited. However, the subsequent addition of the correct proportion of troponin B results in the complete recovery of ATPase activity, which indicates a preferred association of the polyanion with troponin B. The addition of polyanion to troponin B and actomyosin may therefore follow two phases: initially at low concentrations the polyanion combines with troponin B and then when the sites on troponin B become saturated, the polyanion combines with myosin. With troponin A, this complication is not evident.

In the presence of both troponin B and tropomyosin, the effect of troponin A or polyanions is significantly different from the calcium-insensitive neutralization of inhibition discussed in the foregoing. In the absence of calcium the inhibition by the troponin B–tropomyosin complex is removed only gradually requiring relatively large amounts of polyanions and is removed even less effectively with troponin A. In the presence of calcium, inhibition is relieved by much lower concentrations of both troponin A and the polyanions. This indicates that calcium regulation can be conferred on the troponin B–tropomyosin complex by troponin A or the polyanions. Again, this effect of troponin A appears to be related to its net negative charge. The polyanionic protein, however, seems to have an added advantage over the negative charge effect introduced for example by RNA, since it allows a more complete persistence of inhibition in the absence of calcium.

These experiments demonstrate clearly that the conferral of calcium regulation requires tropomyosin in addition to troponin B and troponin A or the polyanions. The obvious question now is where does calcium bind? Several groups have found that calcium binds to troponin (Ebashi et al., 1967; Fuchs and Briggs, 1968; Drabikowski et al., 1968; Arai and Watanabe, 1968). Some binding to tropomyosin has been reported, although it was considerably less than 1 mole of calcium/mole of tropomyosin and it was suggested that this
was a result of contamination by troponin (Fuchs and Briggs, 1968). We have found that troponin A binds calcium. It has not been determined whether or not RNA under similar conditions binds significant amounts of calcium. However, if the effect of calcium is due to its binding to either troponin A or the polyanions, then this effect is not due to a charge neutralization by Ca++.

The reason for this is that much less polyanion or troponin A is needed to remove the inhibition by troponin B and tropomyosin in the presence of calcium, where the net negative charge might be expected to be reduced compared to the situation in the absence of calcium. Binding of calcium to troponin B was also found, but less than troponin A on a weight basis. Precise binding parameters are being determined. The regulatory mechanism may therefore depend on calcium bound to both troponin A and troponin B. The involvement of two classes of calcium-binding sites in the regulation of ATPase activity in intact myofibrils has recently been shown by Weber (1969a). Ebashi et al. (1968) also found that their calcium-binding data for troponin were best explained by assuming two distinct binding sites.

Whatever may be the outcome of the calcium-binding experiments with RNA and heparin, it is unlikely that they will adequately explain the effects produced by the polyanions. Extensive binding of Ca++ to saturate the charged groups of the polyanion would eliminate the negative charge. A smaller extent of binding to only a few sites would require that these sites be specifically located, an unlikely premise in view of the structures of RNA and heparin. Furthermore, it is equally unlikely that a similar structural change which could be transmitted to troponin B could be elicited on the different polyanions by calcium binding. The hypothesis may, therefore, be suggested that calcium sensitivity is introduced with RNA or heparin not as a result of calcium binding to the polyanion, but rather as a consequence of the interaction of this polyanion with troponin B. It is possible that the conformation of troponin B is altered by combination with the polyanion and that this change allows a calcium-dependent response, so that it is only an inhibitor in the absence of calcium. As already indicated, the presence of tropomyosin would also be required to realize effective inhibition.

The reason why tropomyosin is essential for calcium regulation seems to be related to the fact that it has an affinity for both actin and troponin B and thus serves to link the regulatory proteins to actin. In this manner the orientation of troponin could be fixed to ensure maximal efficiency of the calcium regulatory mechanism. It has been proposed that the distribution of troponin on the thin filament reflects an underlying periodicity of tropomyosin (Ebashi and Endo, 1968; Ebashi et al., 1968). If the binding of calcium to troponin causes a conformational change as Ebashi et al. (1968) have suggested, then tropomyosin could function as an intermediary in the transmission of the effect to actin. Indeed Tonomura et al. (1969) have recently shown that a calcium-
dependent structural effect can be detected, and that this is transmitted from troponin through tropomyosin to actin. There are also indications that tropomyosin qualitatively changes the behavior of troponin; e.g., in the presence of calcium troponin plus tropomyosin activates the ATPase activity whereas under the same conditions troponin alone inhibits slightly. Whether these differences are due merely to the binding of troponin B to tropomyosin, or whether the interaction of tropomyosin with troponin B alters the latter cannot at the moment be decided.

Other features to be incorporated into a more complete mechanism must also include the effect of nucleoside triphosphate binding to myosin. Especially significant are the observations made recently by Weber, who found that relaxation depends on a relationship between the extent of calcium binding by troponin and ATP binding by myosin (Weber, 1969 a) and also that several nucleoside triphosphates are capable of effecting relaxation of myofibrils (Weber, 1969 b). These results indicate that the interactions of tropomyosin and troponin A and B are not sufficient to define completely the resultant actomyosin ATPase activity but that this depends in addition on the state of myosin.

In summary the major features of the above study should be emphasized. Troponin B is an inhibitor of ATPase activity and this inhibition can be relieved by troponin A, an effect apparently depending on the polyanionic nature of troponin A since other polyanions behave similarly. Calcium sensitivity, however, requires the presence of tropomyosin in addition to troponin A and troponin B. Without tropomyosin, troponin A removes the inhibition by troponin B in both the presence and absence of calcium. With tropomyosin, the relief of inhibition by small quantities of troponin A occurs only with calcium present. On the other hand without troponin A, the inhibition of ATPase activity by troponin B is calcium insensitive and the addition of tropomyosin although enabling a more effective inhibition by troponin B is not sufficient to restore the regulation by calcium.

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