Troponin-Tropomyosin Complex

COLUMN CHROMATOGRAPHIC SEPARATION AND ACTIVITY OF THE THREE ACTIVE TROPONIN COMPONENTS WITH AND WITHOUT TROPOMYSIN PRESENT*

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SUMMARY

By means of new or modified procedures troponin-tropomyosin complex was fractionated into three components plus tropomyosin, and then reconstituted in the absence of urea. Tropomyosin was separated from troponin by hydroxypatite column chromatography, a method giving sharper separation than the previously used technique of isoelectric precipitation. The chromatography also separated the tropomyosin into two fractions. One gave a single band on sodium dodecyl sulfate gel electrophoresis, and the other a double band; and the complete absence of proline suggested that each was a different form of pure tropomyosin. Tropomysins I, T, and C were separated by DEAE-Sephadex chromatography in 6 M urea as previously, but the separation was found to be much improved if the troponin was treated with a Ca\(^{2+}\) chelator before being applied to the column. The activity of the troponin fractions was assayed at varying ratios to actin both with and without tropomyosin present. It was found that near physiologic ratios to actin, either the combination of troponin I plus tropomyosin or troponin I plus troponin T inhibited the acto-heavy meromyosin ATPase. However, in both cases when troponin C, which is necessary for Ca\(^{2+}\) sensitivity, was added, the inhibition was reversed not only in the presence but also in the absence of Ca\(^{2+}\). Only when both troponin T and tropomyosin were present in addition to troponin I and C did inhibition occur in the absence but not in the presence of Ca\(^{2+}\). We therefore conclude first, that if troponin T is present tropomyosin may not be required for inhibition by troponin I at physiologic ratios to actin, second, that all three components plus tropomyosin are necessary to restore full Ca\(^{2+}\) sensitivity, and third, that this reconstitution can be accomplished by combining the individual components in the absence of urea.

In 1963 Ebashi first proposed that the relaxation of skeletal muscle depended on the presence of a protein complex which he called “native tropomyosin” (1, 2). In the absence of Ca\(^{2+}\), this protein causes muscle to relax, apparently by preventing the binding of myosin to actin (3, 4), whereas in the presence of Ca\(^{2+}\), it allows contraction to occur.

Since 1963, numerous workers have attempted to characterize the components of native tropomyosin. In 1965, Ebashi and Kodama showed that native tropomyosin was, in fact, composed of two proteins, tropomyosin B, itself, and a new protein which they called troponin (5, 6). More recently, Hartshorne and Mueller showed that troponin was itself composed of at least two proteins which they named troponin A and troponin B (7). A similar separation with the use of SE-Sephadex chromatography in 6 M urea was accomplished somewhat later by Schaub and Perry (8). Even more recently Drabikowski and his coworkers showed that troponin could in fact be separated into four components by fractionation on DEAE-Sephadex A-50 in 4 M urea with ethylenediaminetetraacetate present (9). In a considerably more detailed study Greaser and Gergely found that by fractionating troponin on DEAE-Sephadex A-50 in 6 M urea they could separate it into one inactive and three active components which they called troponin I, troponin T, and troponin C (10). With tropomyosin present, troponin I inhibited the interaction of actin and myosin but showed no Ca\(^{2+}\) sensitivity; troponin C blocked the action of troponin I, but still no Ca\(^{2+}\) sensitivity was observed. Only with all three components present could the full activity of the original troponin be restored. However, the three components had to be recombined in the presence of 6 M urea, which suggested to the authors that the troponin components could not refold into their native configuration in the absence of urea.

In the present study we developed new methods of separating both tropomyosin from troponin and the three troponin components from each other. By using hydroxyapatite chromatography, we were able to separate the troponin and tropomyosin more effectively than by the previously used method of isoelectric precipitation (11). Furthermore the hydroxyapatite chromatography separated the tropomyosin into two fractions, one of which gave a single band on sodium dodecyl sulfate gel electrophoresis, and the other a double band; and the complete absence of proline suggested that both might be isomers of tropomyosin.

We also found that separation of the three components of...
troponin on DEAE-Sephadex could be considerably improved if before chromatography the troponin was exposed to EDTA or EGTA. In this way three cleanly separated components could be obtained. The effect of these three components on the acto-heavy meromyosin adenosine triphosphatase was studied at varying ratios to actin, both with and without troponymosin present. Our results suggest that troponymosin may not be required for inhibition of the acto-heavy meromyosin ATPase, since the complex of troponin I and T causes almost 90% inhibition near physiologic ratios to actin even without tropomyosin present. On the other hand, for full Ca$^{2+}$ sensitivity to occur varying ratios to actin, both with and without tropomyosin present. Our results suggest that tropomyosin may not be required for inhibition of the acto-heavy meromyosin ATPase, since the complex of troponin I and T causes almost 90% inhibition near physiologic ratios to actin even without tropomyosin present. On the other hand, for full Ca$^{2+}$ sensitivity to occur.

\[ \text{PROTEIN PREPARATIONS--Myosin was prepared by the method of Kielley and Harrington (12) and heavy meromyosin was prepared from the myosin as previously described (13). Actin was prepared either by the method of Adelstein (14) or by a modified method of Spudich and Watt (15). In this latter method the actin was treated twice with 0.8 M KCl rather than just once with 0.6 M KCl.} \]

\[ \text{Troponin-tropomyosin complex was prepared by the method of Hartshorne (11) as follows. Myofibrils were prepared by the method of Perry and Zdyrko (16), including washing 7 to 10 times with buffer. The myofibrils were extensively washed in this manner in an effort to remove proteolytic enzymes which might degrade the troponin during later stages of purification. Following the washing, the myofibrils were dissolved in 0.6 M KCl and the resulting actomyosin gel precipitated by dilution. It was then washed with alcohol and ether, and finally air-dried. The resulting powder could be stored at --20° for as long as 6 months without any change being noted.} \]

\[ \text{The troponin-tropomyosin complex was extracted from the actomyosin powder according to the method of Hartshorne (11). Extraction was carried out overnight with 1 M KCl, the resulting extract dialyzed against 4 volumes of water, and then the 40 to 60% (NH$_4$)$_2$SO$_4$ fraction isolated. This fraction will be called troponin-tropomyosin complex.} \]

\[ \text{The troponin and tropomyosin were separated by means of hydroxyapatite chromatography. The hydroxyapatite was either made in our laboratory by a modified method of Levin (17) or was purchased from Bio-Rad in the wet form. Both gave the same separation. The hydroxyapatite was equilibrated in 1 M KCl, 1 mM PO$_4$ at pH 7.0, 2 mM DTT. Two to three hundred milligrams of troponin-tropomyosin in 1 M KCl, 2 mM imidazole, 5 mM 2-mercaptoethanol in a volume of 20 to 30 ml was applied to a column (2.5 X 25 cm) which was eluted with a linear PO$_4$ gradient from 2 M KCl, the resulting actomyosin gel precipitated by dilution. It was then washed with alcohol and ether, and finally air-dried. The resulting powder could be stored at --20° for as long as 6 months without any change being noted.} \]

The troponin eluted from the column was concentrated by Amicon filtration and was then dialyzed against two changes of 0.5 M KCl. 2 mM imidazole, 2 mM DTT, 2 mM EDTA, and then for 48 hours against 6 M urea, 50 mM Tris, 2 mM DTT, 1 mM EDTA at pH 8.0. It was then chromatographed on a DEAE-Sephadex A-50 column. The DEAE-Sephadex A-50 was swelled in 50 mM Tris at 25° and then equilibrated at 0° (to prevent cation formation) with 6 M urea, 50 mM Tris, 1 mM DTT at pH 8.0. No EDTA was added to the column. Tropomin (60 to 70 mg) in 6 M urea, 50 mM Tris, 2 mM DTT, 1 mM EDTA at pH 8.0 in a volume of 6 to 7 ml was applied to a column (0.9 X 60 cm) which was eluted with a linear KCl gradient from 6 M urea, 50 mM Tris, 2 mM DTT, 0.6 M KCl, 6 M urea, 50 mM Tris, 2 mM DTT (total elution volume 100 ml). The column was run by gravity flow at a pressure head of 30 cm with a flow rate of 4 to 5 ml per hour and two samples per hour were collected. Following elution the fractions were dialyzed to remove the urea, the troponin C against 2 mM imidazole, 2 mM 2-mercaptoethanol and the troponin I and tropomelin against 0.3 M KCl, 2 mM imidazole, 2 mM 2-mercaptoethanol. These solutions could be stored for at least 1 week at 0° without any noticeable change in activity.} \]

\[ \text{RESULTS--Separation of Tropomyosin on Hydroxyapatite--When 200 to 300 mg of troponin-tropomyosin complex were applied to a hydroxyapatite column and eluted with a phosphate gradient in 1 M KCl, as shown in Fig. 1, three major fractions were obtained. Fraction III, which was identified as troponin will be discussed first.} \]

\[ \text{It was eluted from the hydroxyapatite as a double peak and the electrophoretic pattern of each part is shown in Fig. 2. The front part gave a single band with a molecular weight of approximately 35,000, whereas the back part gave a double band with molecular weights of approximately 35,000 and 31,000. Rechromatography of the back part alone did not further fractionate it. Rather it still ran as a double band on gel electrophoresis. It has previously been reported that tropomyosin occurs as a double band on gel electrophoresis (10, 18), and we considered the possibility that the heavier component was due to contaminant troponin T which has a similar molecular weight (10). However, amino acid analysis showed that troponin T contains a considerable amount of proline, whereas both the front and back parts of the tropomyosin peak showed the complete absence of proline. Since the heavy component was a significant fraction of the back part, it clearly could not be troponin T. Therefore, it appears that skeletal muscle tropomyosin is made up of two fractions, one of which contains two nonidentical polypeptide chains. This conclusion is consistent with other recent studies on tropomyosin which suggest that the tropomyosin is heterogeneous (22-24). In the experiments described (13) by ultraviolet absorption at 278 or 280 nm. The extinction coefficients employed were 380 cm$^9$ per g at 278 nm for tropomyosin and 450 cm$^9$ per g at 278 nm for troponin (11). For the troponin components they were 590 cm$^9$ per g at 280 nm for troponin I, 504 cm$^9$ per g at 280 nm for troponin T and 200 cm$^9$ per g at 280 nm for troponin C. For determination of molecular concentrations, the following molecular weights were used: troponin I, 24,000; troponin C, 21,000; troponin T, 35,000; tropomyosin, 70,000 (10); and actin, 42,000 (21).} \]

Ammonium sulfate and urea used were Schwarz-Mann "Ultra-pure" reagents.

\[ \text{RESULTS--Separation of Tropomyosin on Hydroxyapatite--When 200 to 300 mg of troponin-tropomyosin complex were applied to a hydroxyapatite column and eluted with a phosphate gradient in 1 M KCl, as shown in Fig. 1, three major fractions were obtained. Fraction III, which was identified as troponin will be discussed first. It was eluted from the hydroxyapatite as a double peak and the electrophoretic pattern of each part is shown in Fig. 2. The front part gave a single band with a molecular weight of approximately 35,000, whereas the back part gave a double band with molecular weights of approximately 35,000 and 31,000. Rechromatography of the back part alone did not further fractionate it. Rather it still ran as a double band on gel electrophoresis. It has previously been reported that tropomyosin occurs as a double band on gel electrophoresis (10, 18), and we considered the possibility that the heavier component was due to contaminant troponin T which has a similar molecular weight (10). However, amino acid analysis showed that troponin T contains a considerable amount of proline, whereas both the front and back parts of the tropomyosin peak showed the complete absence of proline. Since the heavy component was a significant fraction of the back part, it clearly could not be troponin T. Therefore, it appears that skeletal muscle tropomyosin is made up of two fractions, one of which contains two nonidentical polypeptide chains. This conclusion is consistent with other recent studies on tropomyosin which suggest that the tropomyosin is heterogeneous (22-24). In the experiments described (13) by ultraviolet absorption at 278 or 280 nm. The extinction coefficients employed were 380 cm$^9$ per g at 278 nm for tropomyosin and 450 cm$^9$ per g at 278 nm for troponin (11). For the troponin components they were 590 cm$^9$ per g at 280 nm for troponin I, 504 cm$^9$ per g at 280 nm for troponin T and 200 cm$^9$ per g at 280 nm for troponin C. For determination of molecular concentrations, the following molecular weights were used: troponin I, 24,000; troponin C, 21,000; troponin T, 35,000; tropomyosin, 70,000 (10); and actin, 42,000 (21).} \]

Ammonium sulfate and urea used were Schwarz-Mann "Ultra-pure" reagents.
FIG. 1 (left). Fractionation of the troponin-tropomyosin complex on hydroxypatite. Preparation of the troponin-tropomyosin complex and the details of the chromatography are given under “Methods.”

TABLE I

Effect of hydroxylapatite fractions on acto-heavy meromyosin ATPase

The conditions were 2 mM ATP, 1 mM MgCl₂, 20 mM KCl, 0.31 mg per ml of heavy meromyosin, 0.2 mg per ml of actin, either 1 mM EGTA or 0.5 mM Ca²⁺ and where added 0.2 mg per ml of tropomyosin, 0.2 mg per ml of troponin, and 0.2 mg per ml of Fraction I. For determining the concentration of Fraction I, the same extinction coefficient was used as for troponin (see “Methods”). In all cases the ATPase of the heavy meromyosin alone, 0.025 μmole of Pi per mg per min, was subtracted from the measured ATPase. 100% ATPase = 0.74 μmole of Pi per mg of heavy meromyosin per min.

<table>
<thead>
<tr>
<th>Fractions added</th>
<th>ATPase</th>
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<tbody>
<tr>
<td></td>
<td>EGTA</td>
</tr>
<tr>
<td>None</td>
<td>%</td>
</tr>
<tr>
<td>Fraction III (tropomyosin)</td>
<td>91</td>
</tr>
<tr>
<td>Fraction II (troponin)</td>
<td>94</td>
</tr>
<tr>
<td>Fraction III + I</td>
<td>59</td>
</tr>
<tr>
<td>Fraction III + II</td>
<td>71</td>
</tr>
<tr>
<td>Original troponin-tropomyosin complex</td>
<td>9</td>
</tr>
</tbody>
</table>

Fractions added

scribed below, the tropomyosin employed was a combination of the two fractions, since we never found any difference in their activity.

Separation of Troponin on Hydroxylapatite—Table I shows the effect on the acto-heavy meromyosin ATPase of the various fractions from the hydroxylapatite column. As can be seen, under these conditions tropomyosin alone had very little effect. Fraction I in combination with tropomyosin also had only a slight inhibitory effect on the acto-heavy meromyosin ATPase, and it was therefore discarded. The multiple peaks which make up Fraction II were all found to have the same activity and therefore were combined into a single fraction. As can be seen, in the absence of tropomyosin, this fraction showed some inhibition of the acto-heavy meromyosin ATPase, but little or no Ca²⁺ sensitivity was observed. On the other hand, when this fraction was mixed with tropomyosin the effect was almost identical with that of the original troponin-tropomyosin complex and it was therefore identified as troponin.

Separation of Troponin Components on DEAE-Sephadex—To separate troponin into its constituent proteins, we essentially adopted the method of Greaser and Gergely (10), who separated the components on DEAE-Sephadex in 6 M urea. However, we found that the separation depended on whether or not Ca²⁺ was present in the sample. Fig. 3 shows the separation which occurs on DEAE-Sephadex when the troponin is exhaustively dialyzed against EDTA before being applied to the DEAE-column. As can be seen, four quite cleanly separated peaks are obtained. On the other hand, as shown in Fig. 4, with Ca²⁺ present in the sample, the separation is not nearly as sharp; in fact, the second peak is almost completely missing. We therefore always treated the troponin with EDTA (or EGTA, which has the same effect), before fractionating it on DEAE-Sephadex.

Activity of Troponin Components in Presence of Tropomyosin—Fraction I from the DEAE-column was discarded because it gave a multicomponent gel pattern which was quite variable. Fractions II, III, and IV, on the other hand, gave single bands on gel electrophoresis, as shown in Fig. 5, and were identified as
troponin I, troponin T, and troponin C, respectively, based on their gel electrophoresis patterns and a preliminary survey of their effect on the acto-heavy meromyosin ATPase in combination with tropomyosin shown in Table II (cf. Greaser and Gergely (10)). As can be seen in both the presence and absence of Ca\(^{2+}\), Fraction II or troponin I almost completely inhibited the acto-heavy meromyosin ATPase, and Fraction IV or troponin C reversed the effect of the troponin I. In neither case was significant Ca\(^{2+}\) sensitivity observed. The combination of troponin I and C variably caused 1.5- to 2-fold activation in the presence of Ca\(^{2+}\), but in no case was significant inhibition observed in the absence of Ca\(^{2+}\), as with the original troponin. On the other hand, with Fraction III or troponin T present, the activity of the original troponin-tropomyosin complex was essentially reconstituted. As with the original troponin, marked inhibition of the acto-heavy meromyosin ATPase occurred in the absence of Ca\(^{2+}\), whereas in the presence of Ca\(^{2+}\), both the original and reconstituted troponin (in combination with tropomyosin) caused activation of the acto-heavy meromyosin ATPase.

In these assays the troponin components were premixed in the assay solution prior to adding the other protein components. The order of addition of the troponin components did not appear to affect the result. However, when both troponin T and tropomyosin were present in a single sample the best results were obtained by mixing the troponin T and other troponin components with actin prior to adding the tropomyosin. When this was not done, the inhibitory effect of the complex was often reduced, possibly because the troponin T and tropomyosin formed an inactive precipitate in the low ionic strength assay solution. Because of this finding our routine order of addition to the assay solution was 1, the troponin components; 2, actin; 3, tropomyosin; 4, heavy meromyosin (which started the reaction).

An excess of troponin C over troponin I and T was used in these assays to be certain that the troponin I was completely saturated with the troponin C. At a 2:1 molar ratio, the effect of the troponin C was maximal, while at a 1:1 ratio it was not

**Table II**

<table>
<thead>
<tr>
<th>Fractions added</th>
<th>ATPase</th>
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<tbody>
<tr>
<td></td>
<td>EGTA</td>
</tr>
<tr>
<td>None</td>
<td>96</td>
</tr>
<tr>
<td>II (Troponin I)</td>
<td>5</td>
</tr>
<tr>
<td>II + III (Troponin I + T)</td>
<td>5</td>
</tr>
<tr>
<td>II + IV (Troponin I + C)</td>
<td>77</td>
</tr>
<tr>
<td>II + III + IV (Troponin I + T + C)</td>
<td>17</td>
</tr>
<tr>
<td>IV (Troponin C)</td>
<td>82</td>
</tr>
<tr>
<td>III (Troponin T)</td>
<td>74 (132)(^a)</td>
</tr>
<tr>
<td>III + IV (Troponin T + C)</td>
<td>51 (102)(^a)</td>
</tr>
</tbody>
</table>

\(^a\) Rate increased as ATP was hydrolyzed; rate in parentheses is highest rate measured.
near physiologic ratios of troponin I to actin, although perhaps on the molecular weights given under “Methods.” Where it was added, the molar concentration of the troponin C was twice that of the other troponin components. In all cases the ATPase of the heavy meromyosin alone, 0.025 amole of Pi per mg per min, was subtracted from the measured ATPase. 100% ATPase = 0.41 amole of Pi per mg per min. a, troponin I + tropomyosin; O, troponin I + C + tropomyosin; b, troponin I + T + tropomyosin; O, troponin I + T + C + tropomyosin.

Fig. 6. Effect of the troponin components plus tropomyosin at varying ratios to actin. Conditions: 2 mM ATP, 1 mM MgCl₂, 1 mM imidazole (pH 7.0), 30 mM KCl, 1 mM EGTA, 0.5 mg per ml of heavy meromyosin, 0.2 mg per ml of actin. The concentrations of the troponin components and tropomyosin were varied and except for the troponin C were kept equimolar to each other based on the molecular weights given under “Methods.” Where it was added, the molar concentration of the troponin C was twice that of the other troponin components. In all cases the ATPase of the heavy meromyosin alone, 0.025 amole of Pi per mg per min was subtracted from the measured ATPase. 100% ATPase = 0.41 amole of Pi per mg per min. a, troponin I + tropomyosin; O, troponin I + C; b, troponin I + T + tropomyosin; O, troponin I + T + C + tropomyosin.

Fig. 7. Effect of the troponin components at varying ratios to actin in the absence of tropomyosin. Conditions were the same as in Fig. 6. The concentrations of the troponin components were varied and except for the troponin C were kept equimolar to each other based on the molecular weights given under “Methods.” Where it was added, the molar concentration of the troponin C was twice that of the other troponin components. In all cases the ATPase of the heavy meromyosin alone, 0.025 amole of Pi per mg per min was subtracted from the measured ATPase. 100% ATPase = 0.45 amole of Pi per mg per min. a, troponin I; O, troponin I + C; b, troponin I + T; O, troponin I + T + C. The circles and triangles represent two different preparations of troponin components.

quite complete. Whether this is due to partial denaturation of the troponin C or to relatively weak binding to the troponin I and T in vitro is not clear at this time.

Effect of Troponin Components and Tropomyosin at Varying Ratios to Actin—The results in Table II suggest that all three troponin components are required for reconstituting the original troponin complex. To be certain of this, however, we investigated at varying ratios to actin, the effect of troponin I alone and in combination with the other troponin components. All of these experiments were performed in the absence of Ca²⁺ since, as shown in Table II, Ca²⁺ has no effect in the absence of troponin C, whereas in the presence of troponin C, Ca²⁺ always has the same effect; it completely abolishes inhibition. As can be seen in Fig. 6a, in the presence of EGTA, troponin I plus tropomyosin completely inhibits the acto-heavy meromyosin ATPase at a molar ratio to actin of about 1:1 (solid circles). Theoretically the maximum effect of the tropomyosin-troponin I mixture should occur at about an 0.15 m ratio to actin (solid circles). However, the binding might not be quite stoichiometric because an equilibrium could be occurring between free and actin-bound troponin I and tropomyosin. At any rate maximum inhibition is occurring at much less than a 1:1 molar ratio of troponin I to actin, so that clearly one troponin I molecule is influencing several actin monomers, as is the case in vitro.

When troponin C is added (Fig. 6a, open circles) the inhibition by the troponin I-tropomyosin mixture is almost completely reversed at troponin I to actin ratios of less than 0.25, i.e. in the range where each troponin I affects several actin monomers. On the other hand, at higher ratios of troponin I to actin, the troponin C seems to have less effect, suggesting that at a 1:1 ratio of troponin I to actin complete inhibition might occur even with excess troponin C present. However, we did not perform detailed investigations at such high ratios because of the possibility that slight cross-contamination of the troponin I with troponin T could give misleading results. It therefore appears that near physiologic ratios of troponin I to actin, although perhaps not at much higher ratios, troponin C reverses the inhibitory effect of the troponin I-tropomyosin mixture not only in the presence but also in the absence of Ca²⁺, so that little Ca²⁺ sensitivity occurs. On the other hand, in the absence of troponin C, although the troponin I-tropomyosin mixture can inhibit the acto-heavy meromyosin ATPase, there is still no Ca²⁺ sensitivity because it is the troponin C which binds Ca²⁺.

These data suggest that the function of troponin T is to prevent troponin C from acting in the absence of Ca²⁺, and the data shown in Fig. 6b confirm this view. In the absence of Ca²⁺, the mixture of troponin I, T, and tropomyosin strongly inhibits the acto-heavy meromyosin ATPase with complete inhibition occurring at a molar ratio to actin of less than 0.2 (solid circles). Apparently stronger binding occurs with troponin T present than with just the mixture of troponin I plus tropomyosin, so that binding between the troponin I, tropomyosin, and actin becomes almost stoichiometric. When troponin C is added to the troponin I-T-tropomyosin mixture, thereby completely reconstituting the original troponin-tropomyosin complex, although the inhibitory effect is somewhat reduced, more than 80% inhibition still occurs at an 0.25 m ratio of troponin to actin (open circles). Therefore, in agreement with the work of Greaser and Gergely (10), we find that in some way the troponin T prevents troponin C from reversing the inhibitory effect of the troponin I-tropomyosin mixture. Whether it does this by increasing the binding of the troponin I-C complex to actin, or by affecting the action of already bound material will require further investigation.

Effect of Troponin Components in Absence of Tropomyosin—These results show that in the presence of troponin C, which is required for Ca²⁺ sensitivity, troponin T must be present for the full effect of troponin to be observed at physiologic ratios to actin. Still remaining, however, is the important question of the role of tropomyosin. The effect of the troponin components in the absence of tropomyosin. Fig. 7a shows the effect of troponin I both with and without troponin C present and as can be seen near physiologic ratios to actin
only a little inhibition occurs with troponin I alone (solid circles) and none with the troponin I-C complex (open circles). As in the case of the troponin I-C-tropomyosin mixture, greater inhibition may occur at higher ratios of troponin I to actin, but we did not investigate this question due to the possibility that small amounts of cross-contamination of the components could confuse the results.

Fig. 7b shows the effect of troponin I plus troponin T in the absence of tropomyosin and here the result is rather surprising. Strong inhibition occurs at an 0.25 M ratio of troponin I and T to actin (solid symbols), an effect similar to that occurring with the troponin I-tropomyosin mixture (cf. Fig. 6a, solid symbols). Analogously, the addition of troponin C reverses the effect of the troponin I-troponin T mixture (open symbols) just as it did the effect of the troponin I-tropomyosin mixture (cf. Fig. 6a, open symbols). This experiment was repeated with several different preparations and the results were always identical; inhibition occurred at relatively low ratios to actin and was reversed by troponin C. It therefore appears that tropomyosin is not necessary for troponin I to inhibit the acto-heavy meromyosin ATPase near physiologic ratios to actin; apparently troponin T can at least partially duplicate the role of tropomyosin in allowing each troponin I molecule to influence several actin monomers. However, both tropomyosin and troponin T are necessary for inhibition to occur in the presence of troponin C; this is why the complete troponin complex, I + T + C, does not itself inhibit the acto-heavy meromyosin ATPase in the absence of Ca\(^2+\).

**DISCUSSION**

The use of hydroxyapatite chromatography to separate troponin and tropomyosin appears to be much more efficient than the previously employed method of isoelectric precipitation. The troponin and tropomyosin are eluted at widely separated points from the column and the column also serves the purpose of removing a small amount of contaminant material from the troponin.

In addition to yielding quite pure troponin and tropomyosin, the hydroxyapatite column also separates the tropomyosin into two fractions, one of which gives a single band and the other which separates into two approximately equal bands on sodium dodecyl sulfate gel electrophoresis. Since neither fraction contains proline and both have the same effect on the acto-heavy meromyosin ATPase, they both appear to be essentially pure tropomyosin. It therefore appears that skeletal muscle contains two tropomyosin fractions, one with identical and the other with nonidentical chains, a result consistent with recent structural studies on tropomyosin (22, 23). Since this work was completed, Cummins and Perry succeeded in separating the light (a) and heavy (b) chains of tropomyosin on CM-cellulose in 8 M urea (24). They were also able to recombine these chains into the three possible isomers of tropomyosin, αα, βb, and αβ, all of which seemed to have identical activities in vitro. In contrast to the situation in 8 M urea, however, in hydroxyapatite in 1 M KC1, although the tropomyosin is monomeric, presumably the two polypeptide chains making up each tropomyosin molecule do not separate. Therefore, hydroxyapatite probably separates the isomers of tropomyosin without rearranging the polypeptide chains, and in this case our results suggest that the αα and αβ isomers of tropomyosin occur in vivo, but the βb isomer does not. To determine why this should be the case and what the role of the isomers of tropomyosin is in vivo will require further work.

In vitro, we have thus far found no difference in the activity of the αα and αβ isomers, but Greaser and his co-workers (26) using our method of separation have reported a difference in their crystal structure under certain conditions.

Our results with the troponin components extend the work of Greaser and Gergely (10) who have also recently modified their procedure with the use of both DEAE-Sephadex and SE-Sephadex (27). We find that the separation which they originally performed on DEAE-Sephadex can be improved considerably if Ca\(^2+\) is first removed from the troponin-tropomyosin complex before it is chromatographed. This result may be related to the recent finding of Tanaka (28) that even in urea, micromolar Ca\(^2+\) concentration can affect the conformation of tropomyosin. The purified troponin components resulting from our preparation each have specific effects on the acto-heavy meromyosin ATPase. In the presence of tropomyosin, troponin I inhibits the acto-heavy meromyosin ATPase both in the presence and absence of Ca\(^2+\). Maximum inhibition occurs at about a 1:4 molar ratio of troponin I to actin, more than the physiologic ratio of about 1:6.5 (25) but still in a range where each tropomycin I is affecting several actin monomers. It is possible that in vitro an equilibrium occurs between free and actin-bound troponin I so that at a 1:4 ratio to actin, only about two-thirds of the troponin I is actually bound, i.e. the ratio of bound troponin I to actin is really 1:6, but direct binding studies will be required to determine if this is in fact the case.

In agreement with Greaser and Gergely (10), we found that adding the Ca\(^2+\)-binding component troponin C to the troponin I and tropomyosin does not restore Ca\(^2+\) sensitivity, at least at physiologic ratios to actin, for with both troponin I and C present the inhibition in the absence of Ca\(^2+\) is reversed. Therefore, inhibition occurs neither in the presence nor absence of Ca\(^2+\). Perry and his co-workers have suggested that tropomyosin plus troponin I and C will restore Ca\(^2+\) sensitivity (29), but Ebashi has found that this restoration occurs only at relatively high concentrations of troponin I and C (30). In agreement with this, we find that at relatively high ratios of troponin I and C to actin almost 50% inhibition of the acto-heavy meromyosin ATPase occurs in the absence of Ca\(^2+\). But near physiologic ratios to actin, where each troponin I affects several actin monomers, it seems quite clear that tropomyosin plus troponin I and C does not restore the activity of the original troponin-tropomyosin complex.

The crucial component necessary for complete restoration of the troponin activity at physiologic ratios to actin appears to be troponin T. When troponin T is added to the troponin I and tropomyosin the binding between the troponin components and actin seems to be somewhat strengthened, since maximum inhibition of the acto-heavy meromyosin ATPase occurs at about a 1:5 ratio of the troponin components to actin. When troponin C is added some reversal of this inhibition occurs, but more than 80% inhibition still occurs at a 1:4 ratio of the troponin to actin, where each troponin is influencing several actin monomers. Therefore, troponin T seems to be able to prevent troponin C from reversing the inhibitory effect of the troponin I in the absence of Ca\(^2+\), while still allowing this reversal to occur in the presence of Ca\(^2+\). The mechanism by which it does this remains unclear. Nevertheless our data confirm that with troponin C present, troponin T as well as troponin I are necessary to restore the activity of the original troponin at physiologic ratios to actin.

Using actomyosin as an assay system, Greaser and Gergely (10) were unable to reconstitute troponin by mixing the components in the absence of urea, although they could reconstitute it by recombining the components in the presence of urea. In our experiments we had no problem reconstituting the troponin
by simply mixing the troponin components together in the low ionic strength assay solution prior to adding actin, and then adding actin prior to adding the tropomyosin (and heavy meromyosin). This order of addition was followed because poor results were obtained when the troponin T and tropomyosin were mixed in the low ionic strength assay solution prior to the addition of actin. Whether this problem occurred in the Greaser and Gergely experiments or whether the problem is that actomyosin was used in the assay is not clear. At any rate we were always able to reconstitute the full activity of the troponin by mixing the components in the absence of urea, so apparently the purified troponin components do not require urea to refold into their native configuration.

All of the data we have obtained with tropomyosin present is consistent with the model of troponin-tropomyosin action which suggests that tropomyosin alone is responsible for blocking the interaction of actin and heavy meromyosin, while the troponin simply orient the tropomyosin on the actin filament and makes the blocking of the troponin Cα3+-sensitive (29). This model is supported by structural studies which show that each tropomyosin molecule lies along seven actin monomers, one-half turn of the actin helix, while each troponin, presumably a more globular molecule, binds to the actin and tropomyosin at a single point in each half-turn of the helix (25). It is also supported by x-ray diffraction studies which show that in vivo the tropomyosin appears to occur in two different orientations on the actin filament, one of which is correlated with relaxation and the other with contraction, with Cα3+ inducing a shift from the former to the latter orientation (31–33).

In its simplest form this model would suggest that in the absence of tropomyosin, the troponin components alone would be unable to inhibit the acto-heavy meromyosin ATPase, particularly at physiologic ratios to actin. However, Perry and his co-workers found that at a 1:1 ratio to actin, troponin I alone was able to inhibit the acto-heavy meromyosin ATPase (29). On this basis they argued that troponin I has a direct effect on actin, with tropomyosin simply mediating this effect when the troponin is present at low ratios to actin. Of course inhibition at a 1:1 ratio to actin may occur by a completely nonphysiologic mechanism, and our results with troponin I alone show that near physiologic ratios to actin, troponin I indeed causes very little inhibition of the acto-heavy meromyosin ATPase. However, the results with troponin T also present are quite different. Here strong inhibition does occur near physiologic ratios to actin where the troponin I and T are clearly affecting several actin monomers. In agreement with this result, Hartshorne and Mueller (7) previously showed that troponin B, a rather crude mixture of troponin I and T, inhibited the acto-heavy meromyosin ATPase in the absence of tropomyosin, but no attempt was made in these experiments to accurately determine the ratio of the troponin components to actin.

The finding that troponin I and T inhibit the acto-heavy meromyosin ATPase at close to physiologic ratios to actin suggests that each troponin IT complex may be able to simultaneously block several actin monomers from interacting with heavy meromyosin, even in the absence of tropomyosin. This inhibitory effect of the troponin I-T complex seems to be very similar to the inhibitory effect of the mixture of troponin I and tropomyosin and, analogously, in both cases troponin C blocks the inhibition not only in the presence but also in the absence of Cα3+. Therefore both troponin T and tropomyosin must be present in addition to troponin I and C, to restore Cα3+ sensitivity. Our results with troponin I and T suggest that the mechanism of action of troponin-tropomyosin may be more complex than simply troponin acting on tropomyosin and tropomyosin in turn acting on actin. Rather the troponin itself may have a direct effect on the actin filament with tropomyosin enhancing and modifying this effect. Of course, it is possible that the effect of troponin I and T we are observing is nonphysiologic. Nevertheless, it does suggest that several alternative models of troponin-tropomyosin action will have to be considered and tested in the future.

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Troponin-Tropomyosin Complex: COLUMN CHROMATOGRAPHIC SEPARATION AND ACTIVITY OF THE THREE ACTIVE TROPONIN COMPONENTS WITH AND WITHOUT TROPOMYOSIN PRESENT
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