FRACTIONATION OF TROPONIN INTO TWO DISTINCT PROTEINS[1]

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Received April 1, 1968

The protein complex which governs the calcium sensitivity of natural actomyosin (Ebashi, 1963) has been shown to consist of two components, tropomyosin and troponin (Ebashi and Kodama, 1966; Hartshorne and Mueller, 1967). In the present study troponin has been separated into two distinct proteins, as judged by amino acid analyses. The fractionation was achieved at low pH and high ionic strength. One of the fractions, termed troponin B, effected a calcium insensitive inhibition of synthetic actomyosin ATPase activity, which was enhanced by the addition of tropomyosin. The other fraction, troponin A, conferred calcium sensitivity to the troponin B - tropomyosin system. The separation was reversible and all the properties of the source troponin were regained upon mixing troponin A and B in the correct proportions.

METHODS

Preparation and fractionation of troponin: Natural actomyosin was prepared as described by Perry and Corsi (1958) and submitted to the Bailey sequence of organic solvents (Bailey, 1948) with the modification that dithiothreitol (2 mM) was included in each solvent. The resultant

[1] Supported by grants #67 714 from the American Heart Association and HE 09544 and FR 558001 from the National Institutes of Health.

Abbreviation: EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N'-tetraacetic acid.

powder was dried and extracted with 1 M KCl, 2 mM dithiothreitol (pH 7.0). A fraction precipitated between 40 and 60% ammonium sulfate saturation contained the tropomyosin and troponin. This fraction in 1 M KCl, 2 mM dithiothreitol was adjusted to pH 4.6 and the supernatant taken as troponin. Tropomyosin was isolated from the precipitate by ammonium sulfate fractionation between the limits of 53 to 60% saturation. The ammonium sulfate and isoelectric fractionations were dependent on the protein concentration, and the fractions described were prepared from source fractions of low concentration (approximately 1 mg/ml). Complete preparative details will be published later.

The troponin was adjusted to 1.2 M KCl, 2 mM dithiothreitol and HCl was added to 0.1 M. The final protein concentration was 4 mg/ml. The acidified mixture was dialyzed overnight against 1.2 M KCl, 2 mM dithiothreitol, 0.1 M HCl. Centrifugation at 100,000 g for 1 hour gave troponin A as the precipitate, which was dissolved and dialyzed against 10 mM Tris-HCl (pH 7.6), 2 mM dithiothreitol. The supernatant was neutralized and dialyzed against the above solvent, and this resulted in the precipitation of between 90 and 95% of the protein. The precipitate, troponin B, was washed with the dialysis solvent and dissolved in 5 mM ATP, 10 mM Tris-HCl (pH 7.6), 2 mM dithiothreitol.

Re-acidification of troponin A and B in 1.2 M KCl resulted in the quantitative precipitation of troponin A, whereas no precipitation occurred with troponin B.

ATPase activity was estimated at 25° using synthetic actomyosin, (4:1 w/w ratio of myosin to actin [Mueller, 1966]) in a basic medium containing 25 mM Tris-HCl (pH 7.6), 2.5 mM $MgCl_2$ and 2.5 mM ATP. For the determination of Ca^{++} sensitivity the Mg^{++} activated ATPase was measured in the presence and absence of 1 mM EGTA. These two assay solutions will be referred to as the Mg^{++} and Mg^{++}-EGTA media.

Protein concentrations were estimated from micro-Kjeldahl nitrogen analyses assuming 16% nitrogen.

Results and Discussion:

The composition of the source troponin was estimated from the yields of the two components to be approximately 0.3 parts of troponin A to 0.7 parts of troponin B (w/w). The reconstitution of source troponin, which was soluble at low ionic strength, was achieved by mixing troponin A and B in these proportions. Recombination was best performed at high ionic strength (1.0 M KCl), where both proteins were soluble, followed by dialysis to low ionic strength (10 mM Tris-HCl [pH 7.6] 2 mM dithiothreitol). The limited solubility of troponin B under the latter conditions therefore was increased by the addition of troponin A. This reversibility of the pH 1 treatment indicated that the separation was not due to acid denaturation, but rather to a selective fractionation of the two components. That two distinct proteins were involved is evident from the amino acid analyses given in Table I, which also indicates that neither troponin A nor troponin B was identical with the source protein.

Amino Acid	Troponin	Troponin A	Troponin B
Lys	99.0	77.3	109.5
His	13.3	8.2	15.7
Arg	63.4	37.0	77.8
Asp	92.1	114.5	80.7
Thr	25.1	36.9	19.8
Ser	36.5	37.2	35.6
Glu	173.4	155.1	180.7
Pro	30.3	34.8	28.4
Gly	44.6	61.9	35.1
Ala	80.5	80.2	80.6
Val	34.8	42.7	29.8
Met	30.1	34.9	27.4
Ileu	31.1	39.9	26.7
Leu	69.2	63.1	72.8
Tyr	12.7	14.6	12.8
Phe	27.2	45.4	19.3

Table I. Amino Acid Composition of Troponin and Its Constituent Proteins (moles amino acid/10^5 g protein)

The solubility of troponin B at low concentrations of ATP (see Methods) was ascribed to the solubilizing effect of the polyanion.

A further distinction between the two fractions was observed for their E_{278}/E_{260} ratios. Representative figures for these were 0.9 and 1.6 for the troponin A and B respectively. The low value obtained for the troponin A may reflect the presence of nucleotide, but at the moment no significance can be attached to this finding.

The troponin constituents were also widely different with respect to their effect on the Mg^{++} activated ATPase activity of synthetic actomyosin (Fig. 1). Troponin A had little effect. Troponin B inhibited the ATPase activity equally in the presence and absence of EGTA. The addition of Ca^{++} to 5 X 10^{-4} M did not affect the inhibition and confirmed that the effect was Ca^{++} insensitive.

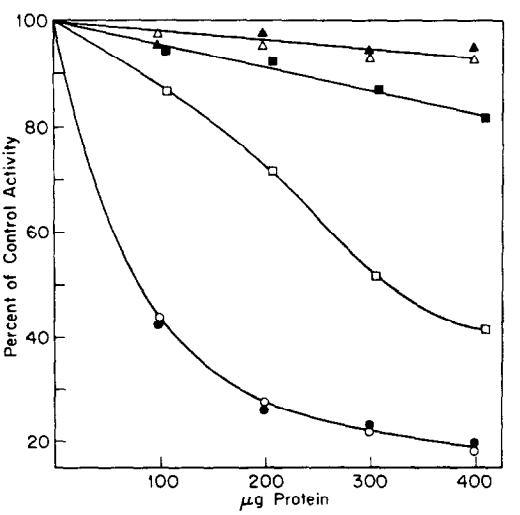

Figure 1. Effect of troponin and its constituent proteins on the ATPase activity of actomyosin. Troponin (□,■), Troponin A (△,▲), Troponin B (○,●). Solid symbols denote assay in Mg^{++} medium. Open symbols denote assay in Mg^{++}-EGTA medium. Actomyosin, 0.32 mg/2 ml.

The inhibition by troponin B however, was reduced by the addition of troponin A. The extent of the reversal was higher in the Mg^{++} than in the Mg^{++}-EGTA medium (Fig. 2). On consideration of the different levels of ATPase activity under the two assay conditions for the source fraction (Fig. 1) this effect would be expected on reconstitution of the parent troponin.

Also it can be seen from Fig. 2 that mixtures of different proportions of troponin A and B resulted in reconstituted troponins exhibiting varying efficiencies for the inhibition of ATPase activity. A low proportion of troponin A may also be reflected by a reduced solubility at low ionic strength.

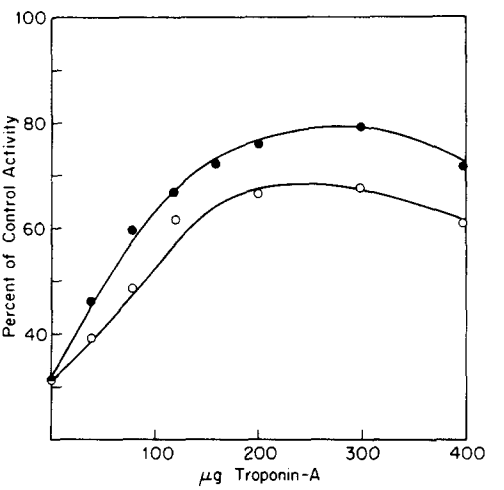

Figure 2. Effect of troponin A on the inhibition caused by a constant amount (200 μg) of troponin B. (●), Mg^{++} medium. (○), Mg^{++}-EGTA medium. Actomyosin, 0.37 mg/2 ml.

The effect of tropomyosin on the ATPase characteristics of troponin A and B is shown in Fig. 3. Relatively little effect was observed with troponin A, although slight activation in the Mg^{++} medium was frequently seen. The addition of tropomyosin to troponin B, however, increased the

extent of inhibition in both the Mg^{++} and Mg^{++}-EGTA assay medias. Maximum inhibition was obtained at approximately a 1:1 w/w ratio. By comparison the addition of tropomyosin to a mixture of troponin A and B (0.3:0.7 parts by weight, respectively) gave the effect associated with the source troponin-tropomyosin interaction, i.e. activation of the actomyosin ATPase activity in the Mg^{++} medium and inhibition in the Mg^{++}-EGTA medium.

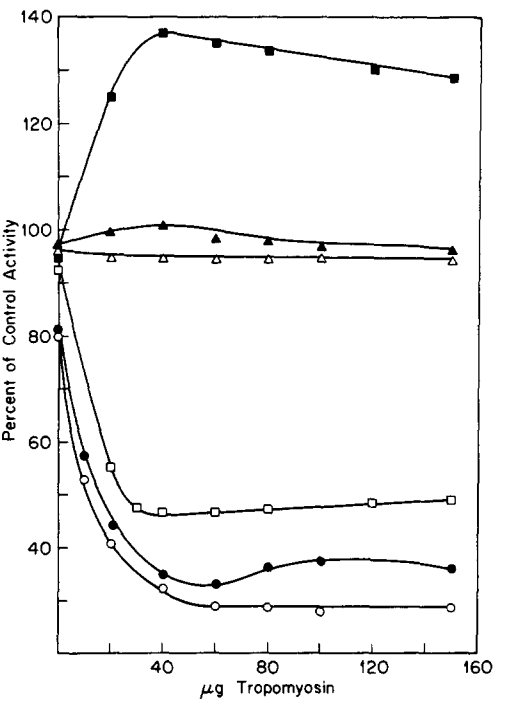

Figure 3. Effect of tropomyosin on the action of troponin A, troponin B, and reconstituted troponin. (○,●), 50 μg troponin B, (△,▲), 100 μg troponin A, (□,■), 50 μg of a 0.3:0.7 w/w mixture of troponin A and B, respectively. Solid symbols denote assay in Mg^{++} medium. Open symbols denote assay in Mg^{++}-EGTA medium. Actomyosin 0.40 mg/2 ml.

Troponin may thus be separated into an inhibitory and an activating principle. The effect which tropomyosin exhibits with the mixture of the two proteins is governed by the free Ca^{++} concentration. Whether this effect is due entirely to protein components and Ca^{++}, or whether an additional factor is involved cannot be determined at this stage.

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