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Erythrocyte Troponin Inhibitor-Like Protein: Isolation and Characterization

Jonathan Maimon and Saul Puszkin

Department of Pathology, Mount Sinai School of Medicine of The City University of New York, 1 Gustave L. Levy Place, New York, New York 10029

A protein was isolated from a human erythrocyte lysate with an apparent molecular weight of 23,000–24,000 daltons. This protein was purified by batch DEAE cellulose followed by column DEAE cellulose chromatography and a gradient of NaCl. On sodium dodecyl sulfate acrylamide electrophoresis, the erythrocyte protein comigrated with muscle troponin inhibitor. An isoelectric precipitation (pH 9.25) was used for the separation of muscle troponin inhibitor from a complex with another troponin component. Both the erythrocyte protein and the muscle troponin inhibitor partially inhibited muscle myosin Ca^{2+} and K^{+}-EDTA ATPase activity. Furthermore, they inhibited actin-activated Mg^{2+}-ATPase of muscle myosin. The inhibitory effects were absent in the presence of muscle troponin calcium-binding component. Muscle troponin inhibitor and the erythrocyte troponin inhibitor-like protein bound to muscle myosin when myosin was precipitated twice at low ionic strength. The presence of a troponin inhibitor-like protein in erythrocytes suggests that it may be a component in the regulation of contractile activity.

Key words: human erythrocyte troponin inhibitor-like protein, muscle troponin inhibitor purification, muscle myosin binding, inhibition of myosin ATPase activity

The regulatory proteins of nonmuscle contractile systems have received a great deal of attention in recent years [1]. The isolation from several nonmuscle tissues of a protein of low molecular weight possessing Ca^{2+}-binding ability and the capacity to confer Ca^{2+} sensitivity to nonmuscle contractile protein interactions has given new insight into the mode by which contractile events are regulated in nonmuscle tissues [2–6]. This new group of Ca^{2+}-modulator proteins, as they are called, are similar to troponin-C (TN-C), the troponin Ca^{2+}-binding protein from skeletal muscle, and are isolated from a variety of tissues including brain [2], platelets [7], erythrocytes [8], and adrenal medulla [9].

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However, a complex system of proteins similar to that of the troponin complex from skeletal muscle has been reported present in platelets [10, 11] and in brain [12]. In human platelets, brain, and smooth muscle, the troponin system probably coexists with another regulatory mechanism associated with the phosphorylation and dephosphorylation of a light chain of myosin [13, 14]. In erythrocytes, the presence of an actin myosin complex has not been established. In its place, an erythrocyte actin-spectrin complex was shown and believed responsible for maintaining the characteristic shape of erythrocytes [15–17].

It was deemed important to determine if other proteins in erythrocytes could regulate contractile activity. We report here the isolation and partial characterization of a protein from erythrocytes which exhibits similarities to those of striated muscle troponin inhibitor (TN-I).

MATERIALS AND METHODS

Purification of Human Erythrocyte TN-I

Red cells were sedimented from whole blood at 5,000g for 6 minutes and the plasma was removed by three washes with 0.154 M NaCl, 1 mM EDTA, and 5 mM Tris-HCl buffer, pH 7.5. Washed erythrocytes were hemolyzed for 20 minutes with two volumes of 5 mM Tris-HCl, pH 7.5, containing 14 mM 2-mercaptoethanol, 5 mM EDTA, and 5 mM EGTA (Buffer-A). The hemolysate was centrifuged at 40,000g for 30 minutes to sediment membranes. The supernatant was used for batch DEAE cellulose chromatography (Whatman Laboratories, Mass.) and equilibrated with Buffer-A in a proportion of 25 gm of dry cellulose/liter of hemolysate and left standing for 24 hours at 4°. The fluid was separated by filtration and the DEAE cellulose fibers washed with three volumes of 1.4 mM 2-mercaptoethanol in 50 mM Tris-HCl, pH 7.5, (Buffer-B). The resin was washed with 0.2 M NaCl in Buffer-B followed by another wash of 0.3 M NaCl in Buffer-B. Proteins were eluted with 0.4 M NaCl in Buffer-B, and the 30–70% $(NH_4)_2SO_4$ saturation pellet dissolved in Buffer-B. The protein solution was dialyzed overnight against Buffer-B and chromatographed on a DEAE cellulose column of 1.5 × 30 cm, equilibrated with Buffer-B. A protein peak was eluted within 0.3 and 0.6 M NaCl in Buffer-B. The 40–70% $(NH_4)_2SO_4$ precipitate was dissolved and dialyzed against Buffer-B. The protein was clarified at 40,000g for 30 minutes and the supernatant used as the erythrocyte TN-I preparation. Biological activity was tested within 48 hours.

Purification of Rabbit Muscle Troponin Components

Rabbit muscle troponin was fractionated by a modification of a previously described method [18]. The protein solution of TN-I and troponin-tropomyosin (TN-T) binding component that emerged unadsorbed from the DEAE cellulose column was adjusted in its pH to 9.25. The resulting precipitate was pelleted at 5,000g for 5 minutes. The protein pellet contained TN-T as the major protein and traces of TN-I. The supernatant consisted of a homogeneous solution of TN-I. Both protein solutions were dialyzed against 0.1 mM $CaCl_2$, 5 mM Tris-HCl, pH 8.0, and stored frozen at −20°.

Preparation of Other Muscle Proteins

Muscle myosin and actin were prepared from fresh rabbit muscle as described elsewhere [19]. Protein concentration was measured by the method of Lowry et al [20], using bovine serum albumin as the standard.

Determination of ATPase Activity

ATPase activities of proteins were determined by the release of inorganic phosphate (Pi) from ATP according to the method of Marsh [21], modified to detect 0.3 nmoles of Pi [22]. Mixtures of gamma-labeled [^{32}P] ATP and nonradioactive ATP were employed. One-half-milliliter aliquots of the butanol layer were counted in a scintillation spectrophotometer. ATPase assays were performed in volumes of 5 ml. Proteins and reagents were mixed initially in a volume of 0.9 ml, incubated at room temperature for 10 minutes, diluted to 5 ml with 50 mM Tris-HCl, pH 7.5, and incubated with ATP at 37° for 10 minutes. For assay purposes, the quantity of protein was 0.01 mg of muscle myosin per ml and 0.01 to 0.02 mg of actin per ml. $CaCl_2$ (10 μM) was added when mixtures of actin and myosin were used. The reaction was initiated by the addition of 0.1 ml of 5 mM gamma-labeled [^{32}P] ATP, 10 mM $MgCl_2$, or 10 mM $CaCl_2$ and allowed to proceed at 37° for 10 minutes. Aliquots of 1 ml were removed at various time intervals. Reactions were stopped by the addition of 0.4 ml 20% trichloroacetic acid. ATPase was estimated as the difference between Pi at zero time and Pi at the end of the reaction.

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis

SDS electrophoresis was performed at room temperature (Buchler Instruments, Inc., New Jersey) on single 7% or 10% polyacrylamide gels containing 10 mM phosphate buffer and 1 gm of SDS per liter [23].

Binding of Proteins to Muscle Myosin

For the determination of binding of muscle and erythrocyte TN-I proteins to muscle myosin, appropriate concentrations of muscle myosin (2 mg/ml) and the inhibitors (2 mg/ml) were mixed and incubated at 37° for 15 minutes in a solution of 0.5 M KCl, 20 mM Tris, pH 7.2. Myosin-inhibitor mixtures were diluted 10 times with distilled water at 4° and the protein precipitate allowed to form by standing 30 minutes at 4°. Proteins were sedimented by centrifugation at 15,000g for 10 minutes. The pellets were dissolved in 0.5 M KCl, 20 mM Tris buffer, pH 7.2, and precipitated again as above. This final precipitate was dissolved in 0.5 M KCl, 20 mM Tris, pH 7.2, and the newly bound protein to myosin molecules was visualized by SDS acrylamide electrophoresis. Binding of TN-I to myosin also was carried out in the presence of TN-C.

RESULTS

For the purification of the human erythrocyte TN-I (see flow chart 1), batch DEAE cellulose chromatography was found suitable to process large volumes of red cell hemolysates. DEAE cellulose adsorbed large amounts of various proteins from the red cell hemolysate. Three washing steps of the DEAE cellulose with Buffer-B and 0.2 and 0.3 M NaCl removed most of the hemoglobin. Several proteins were eluted by 0.4 M NaCl, among them 2 major bands in the 100,000 and 24,000 molecular-weight regions. By DEAE cellulose column chromatography, most of the proteins eluted as one peak (Fig. 1). The inhibitor emerged in the tail portion of the peak as illustrated in Figure 2, when NaCl concentration ranged from 0.35 to 0.6 M.

A 40–70% $(NH_4)_2SO_4$ cut permitted concentration of this protein by precipitation and solubilization at a higher protein concentration, about 2–3 mg/ml. The TN-I preparation showed a 24,000 molecular-weight band. Attempts to isolate TN-I from erythrocyte membranes gave negative results.

PURIFICATION PROCEDURES OF HUMAN ERYTHROCYTE TROPONIN INHIBITOR

Whole Blood

Packed Erythrocytes

Wash 3 x with saline

Centrifuge

Washed Erythrocytes

Hemolyze with 2 volumes

5 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA (pH 7.5)

Centrifuge 40,000 x g for 30 minutes

Hemolyzate

Batch DEAE cellulose (25 gr/L)

Stand for 24 hours

1) Wash with 50 mM Tris-HCl (pH 7.5)

2) Wash with 0.2 M NaCl, 50 mM Tris-HCl (pH 7.5)

3) Wash with 0.3 M NaCl, 50 mM Tris-HCl (pH 7.5)

4) Elute with 0.4 M NaCl, 50 mM Tris-HCl (pH 7.5)

0.4 M NaCl Eluate

30-70% Ammonium sulfate cut

Dissolve in 50 mM Tris-HCl (pH 7.5)

Dialyze, column DEAE cellulose chromatography

Wash with 50 mM Tris (pH 7.5)

Elute with 0-0.6 M NaCl, 50 mM Tris-HCl (pH 7.5)

0.3-0.6 M NaCl fraction

40-70% ammonium sulfate cut

Dialyze against 50 mM Tris-HCl (pH 7.5)

Centrifuge 40,000 x g for 30 minutes

RBC-TN-I supernatant solution

Flow chart 1.

Purification of The Troponin Components From Muscle

The purification procedure used for muscle troponin is similar to that described previously [18] (see flow chart 2). It was found, however, that by DEAE cellulose chromatography equilibrated with 8 M urea and 50 mM Tris-HCl, only TN-C can be separated as a purified protein, while TN-I eluted as a complex with TN-T. When the complex of TN-I and TN-T was adjusted to pH 9.25, a precipitate was formed within 1–2 seconds which consisted of TN-T with small amounts of TN-I. Most of the TN-I remained in the supernatant. Solubilization and precipitation at pH 9.25 for a second time gave a purer TN-T protein solution. Figure 2 shows the purity and electrophoretic mobility of the erythrocyte inhibitor and muscle TN-I.

Binding of Troponin to Muscle Myosin

To determine the affinities of TN-I for myosin, myosin was precipitated at low ionic strength after 15 minutes of incubation with the inhibitor proteins. It was found that for

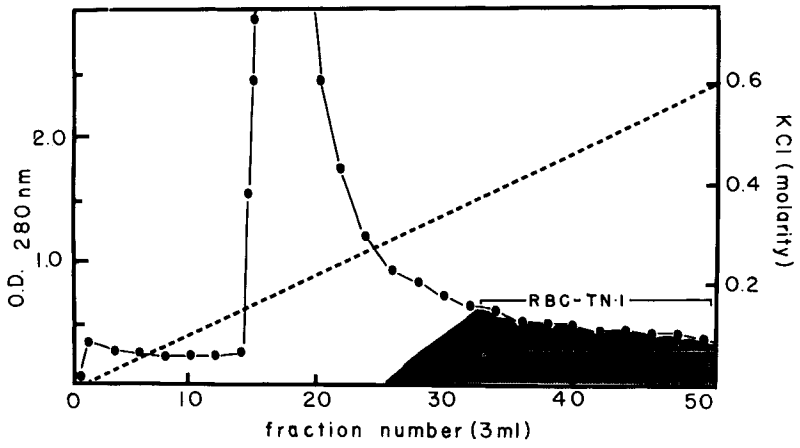


Fig. 1. DEAE-cellulose column chromatography for the purification of the erythrocyte troponin inhibitor-like protein. The 0.4 M NaCl eluate from batch DEAE cellulose (approximately 75 mg of proteins) was loaded on a 1.5 X 30 cm column. The column was washed with 200 ml of buffer and eluted with 150 ml of a continuous gradient of 0–0.6 M NaCl. The shaded area is where the erythrocyte inhibitor eluted.

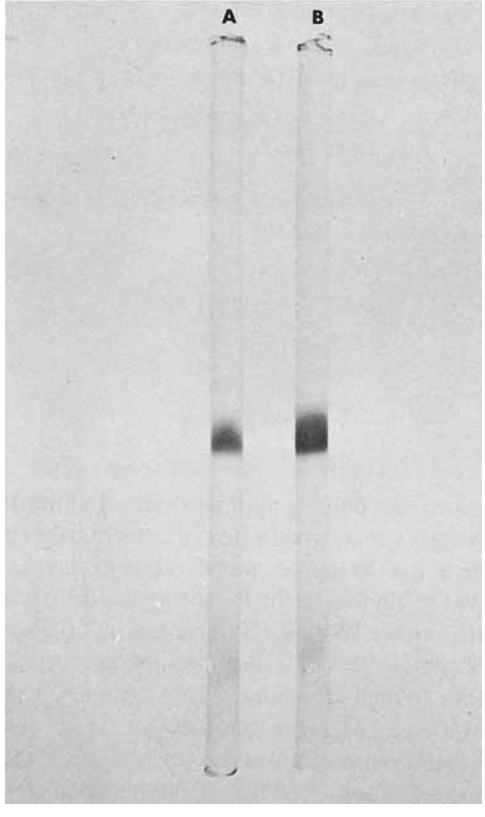
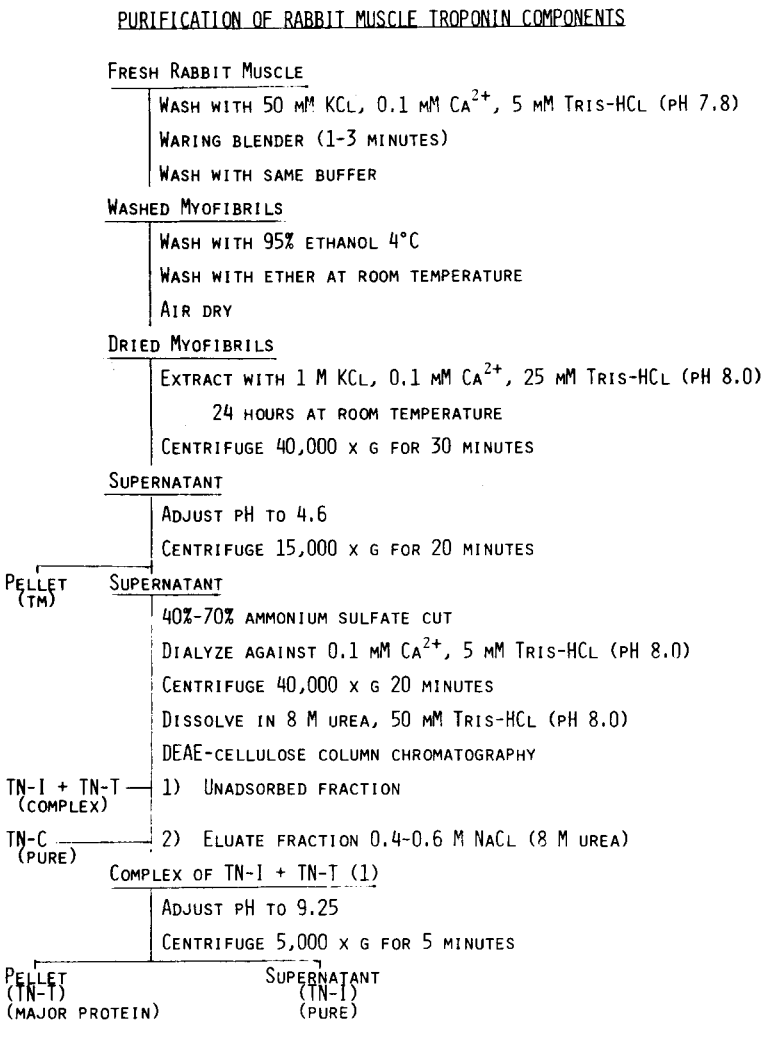


Fig. 2. SDS acrylamide electrophoresis of muscle and erythrocyte TN-I-like protein. Gel A: Muscle TN-I (25 μg). Gel B: Erythrocyte TN-I-like protein (45 μg). Both proteins were loaded on top of single 10% polyacrylamide gels.



Flow chart 2.

myosin, after solubilization, a second precipitation and solubilization again contain a new band bound at equimolecular amounts with that of myosin light chains. By densitometry, the area of the new band bound to myosin was the same as the sum of myosin light chains. Figures 3 and 4 illustrate the binding of the muscle and erythrocyte inhibitors of muscle myosin. Gel A of Figure 3 shows TN-I. Gel B illustrates myosin heavy chains and its three light chains (LC-1, LC-2 and LC-3). Gel C illustrates the polypeptide composition of myosin after allowing muscle TN-I to bind. The muscle TN-I exhibited a relative mobility slightly greater than the LC-1 of myosin. Figure 4 shows similar results obtained with the erythrocyte TN-I. Gel A contains myosin; Gel B contains the erythrocyte TN-I; and Gel C contains bound inhibitor to myosin. The band of TN-I migrated immediately below the LC-1 of myosin. TN-I did not bind in the presence of TN-C.

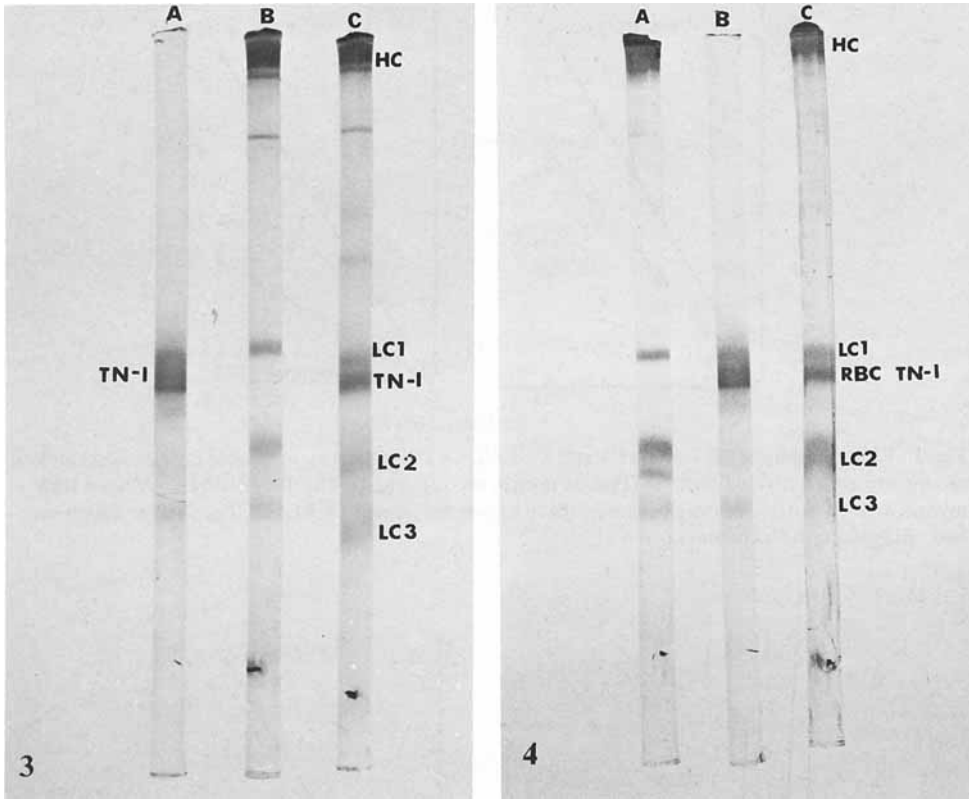


Fig. 3. SDS acrylamide electrophoresis of muscle TN-I bound to muscle myosin. All proteins were loaded on top of single 10% polyacrylamide gels. Gel A: Muscle TN-I. Gel B: Muscle myosin. Gel C: Twice-precipitated muscle myosin after preincubation with muscle TN-I. Bound TN-I migrated slightly below the LC-1 of myosin.

Fig. 4. SDS acrylamide electrophoresis of muscle myosin and erythrocyte TN-I-like protein bound to muscle myosin. All proteins were loaded on top of single 10% polyacrylamide gels. Gel A: Muscle myosin. Gel B: Erythrocyte TN-I-like protein. Gel C: Twice-precipitated muscle myosin after preincubation with erythrocyte inhibitor. Bound erythrocyte inhibitor migrated slightly below the LC-1 of muscle myosin.

ATPase Activity

The three types of cation-activated ATPase activities exhibited by myosin were tested with and without the inhibitor. It was found that myosin Ca^{2+}-ATPase activity at low KCl concentrations was inhibited 30–60% when the inhibitor was present. Figure 5 illustrates the results of such experiments. ATPase activity at 0.5 M KCl and 1 mM EDTA (K^+-EDTA) is inhibited 50% in the presence of TN-I. The Mg^{2+}-ATPase activity of myosin activated by actin was inhibited up to 60% in the presence of TN-I even when 10 μM Ca^{2+} was present in the medium. Figure 6 shows similar effects obtained by the red cell inhibitor on Ca^{2+}-, K^+-EDTA-, and Mg^{2+}-ATPase activities of muscle myosin. Inhibition ranged from 30–50% on myosin Ca^{2+}- and K^+-EDTA-ATPase activities and up to 70% on the Mg^{2+}-ATPase activity of myosin stimulated by actin.

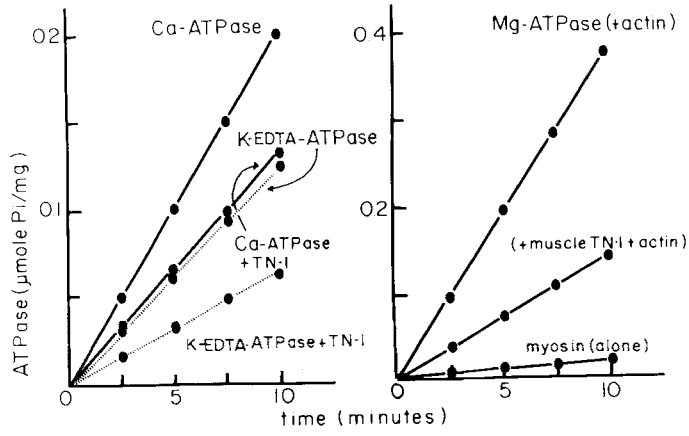


Fig. 5. Effect of muscle TN-I on Ca^{2+}- and K^{+}-EDTA-ATPase activity of muscle myosin (left) and on the muscle actin-activated Mg^{2+}-ATPase of muscle myosin (right). The TN-I was preincubated with myosin at 37°C for 15 minutes before addition of gamma-labeled [^{32}P] ATP. For further details see text. Representative experiment (n = 10).

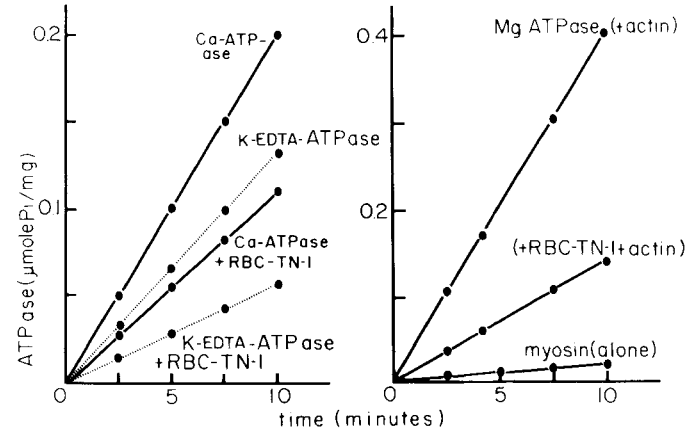


Fig. 6. Effect of erythrocyte TN-I on Ca^{2+}- and K^{+}-EDTA-ATPase activity of muscle myosin (left) and on the muscle actin-activated Mg^{2+}-ATPase of muscle myosin (right). Experimental conditions were the same as those described in legend for Figure 5. Representative experiment (n = 10).

Effects of TN-C on Inhibitor Proteins

In order to determine if erythrocyte TN-I possessed affinities for TN-C similar to those reported for muscle troponin, ATPase activities were performed with mixtures of TN-I and TN-C on myosin and on myosin-actin mixtures containing 10 μM Ca^{2+}. Muscle TN-C and the erythrocyte inhibitor were allowed to react in solution for 10 minutes before starting ATPase activity by the addition of Mg^{2+}-ATP. Figure 7 illustrates the results of such experiments. Myosin shows little Mg^{2+}-ATPase activity. Upon addition of actin, Mg^{2+}-ATPase activity is increased. TN-C produced a small increase in the rate of ATPase activity while addition of TN-I, as expected, showed inhibition. When both proteins,

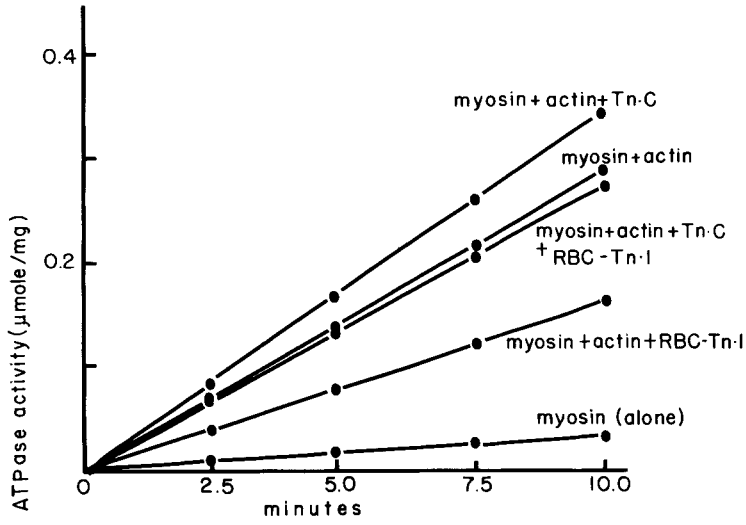


Fig. 7. Effect of muscle TN-C and erythrocyte TN-I-like protein on the muscle actin Mg^{2+}-activated ATPase of muscle myosin. Experimental conditions were the same as those described in legend for Figure 5 except that muscle TN-C was added together with the erythrocyte inhibitor. Representative experiment (n = 10).

TN-C and TN-I, were added the rate of ATPase activity was similar to that of myosin and actin. Both the inhibitory activity of TN-I or the slight activation by TN-C were found cancelled.

DISCUSSION

From human erythrocytes a protein has been isolated with characteristics similar to those of TN-I of muscle. This protein apparently is present in the red cell cytoplasm. Upon rupture of the erythrocyte membrane the inhibitor protein appeared in the hemolysate. The inhibitor – after purification by batch and column DEAE cellulose chromatography – was found to bind to myosin, to inhibit myosin ATPase activity, and to migrate like muscle TN-I on SDS acrylamide gels. Furthermore, it prevented actin from fully stimulating the Mg^{2+}-ATPase activity of myosin. In the presence of muscle TN-C and 10 μM Ca^{2+}, the erythrocyte protein did not show inhibitory activity.

Binding of the erythrocyte inhibitor protein was evident after solubilizing and precipitating myosin at low ionic strength. This effect of muscle TN-I has not been reported previously. In myofibrils it is known that TN-I modulates actin and myosin interaction while attached to the thin filaments of actin through the TN-T component of troponin and tropomyosin [24]. On the other hand, evidence has been presented that actin-myosin interaction in vitro can be modulated by a Ca^{2+}-modulator protein from brain and a muscle TN-I in the absence of TN-T [4]. The effects of TN-I reported in this work may be due to an affinity expressed by TN-I for myosin sites, ie for its light chains, in the absence of TN-T and tropomyosin. In the presence of TN-T and tropomyosin, the inhibitory activity of troponin most likely would occur through the thin filaments.

Although unlikely, the possibility that TN-I binds unspecifically to myosin should be considered. Binding was performed with myosin precipitated from solution at low ionic strength. In the presence of TN-I, both proteins were found in the precipitate. However,

a solution of TN-I showed no precipitation at low ionic strength. These considerations together with the effects of TN-I on myosin ATPase suggest that true binding of the inhibitor to the myosin molecule is the most likely explanation for the inhibitor activity.

The muscle TN-I was separated from TN-T by isoelectric precipitation because the elution of troponin components from DEAE cellulose column chromatography equilibrated with 8 M urea resolved only TN-C while TN-I and TN-T emerged together. Similar results were reported by other investigators [18,24]. An earlier report [25] found that washes of actomyosin gels with low ionic strength buffers and alkaline pH resulted in desensitized actomyosin, probably by removing the components of troponin or by denaturation. When a solution of TN-I and TN-T – dialyzed to remove most of the urea – was adjusted to pH 9.25, a precipitate formed within 1–2 seconds. The supernatant contained TN-I of high purity. The precipitate dissolved easily in neutral pH buffers and contained TN-T with small amounts of TN-I. A second precipitation at pH 9.25 yielded a homogeneous TN-T preparation.

Since erythrocytes apparently do not contain a protein with characteristics similar to those of TN-T of muscle, it was unnecessary to perform DEAE chromatography in the presence of 8 M urea. To resolve the troponin-like inhibitor of erythrocytes from other proteins of the red cell hemolysate, regular DEAE cellulose column chromatography was used with a low ionic strength buffer followed by a NaCl gradient. DEAE chromatography rendered a single peak containing most of the protein present in the erythrocyte hemolysate and permitted elution of erythrocyte TN-I in the tail portion of the peak.

The erythrocyte protein showed inhibitory activity over a wide range of concentration. At molecular ratios of inhibitor to myosin of 0.5 to 5, inhibitory activity was observed. ATPase activity was reduced from 30–60% to that exhibited by muscle myosin alone. Preincubation of the protein for 10 minutes at 37° was sufficient to detect inhibitory activity. It is possible that a relationship exists between the inhibitor protein and the Ca^{2+}-modulator protein since in the presence of TN-C from muscle and 10 μM Ca^{2+}, the inhibitor protein shows no effect on myosin ATPase. Attempts made to isolate the erythrocyte Ca^{2+}-modulator protein have yielded preparations contaminated with other proteins and inadequate at present to test its biological activity.

The physiological role of the protein inhibitor present in erythrocytes remains to be elucidated. A Ca^{2+}-modulator protein with characteristics similar to those of TN-C – or C^{2+}-modulator proteins from other tissues – has been reported present in erythrocyte [8]. These proteins not only modulated the Ca^{2+} sensitivity of actomyosin mixtures, but also activated phosphodiesterase activity [5]. The erythrocyte inhibitor may interact with the modulator protein in vivo for certain red cell functions.

Erythrocytes have been found not to possess an actin myosin system similar to that found in other nonmuscle tissues; however, erythrocytes contain spectrin attached to the cytoplasmic side of the membrane, which to a certain degree exhibits properties similar to those of smooth muscle myosin [15]. Spectrin has been found to complex with actin [15, 26], to influence the polymerizing activities of erythrocyte actin [16, 27], and to possess antigenic determinants common to smooth muscle myosin [15]. It is possible that the erythrocyte inhibitor may play a role in the interaction between erythrocyte actin and spectrin and indirectly regulate specific properties of the erythrocyte membrane; this aspect is being investigated currently. It is significant, however, that erythrocytes contain a protein capable of altering the interacting activity of muscle proteins. Presently, it is not known if the erythrocyte inhibitor exists in the intact erythrocyte, free in its cytoplasm

or attached to actin or spectrin polymers. Likewise, it is not known if, during the separation of proteins, during the formation of a hemolysate, and in the presence of divalent cation chelator, conditions are such that release of this protein from a given site at or near the membrane allows its isolation from the erythrocyte hemolysate.

Recently it was reported [17] that actin plays an important role in the integrity of the erythrocyte membrane and in the shape of the erythrocyte resealed ghosts. Such an inhibitor could provide regulation of actin attachment to membrane sites. Undoubtedly, further study should elucidate its role in red cell functions.

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