PHYSICO-CHEMICAL STUDIES ON THE LIGHT CHAINS OF MYOSIN
III. EVIDENCE FOR A REGULATORY ROLE OF A RABBIT MYOSIN LIGHT CHAIN

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Received February 19,1974

Summary: Treatment of myosin with DTNB causes a decrease in the calcium sensitivity of actomyosin, concurrently with the release of the DTNB light chains. The removal of the calcium-binding DTNB light chains is accompanied by a loss of the calcium binding capacity of myosin. A regulatory role is ascribed to these light chains.

Sulfhydryl reagents are known to influence the Ca^{2+} sensitivity of natural actomyosin (1). This has been ascribed to conformational changes induced in the myosin molecule as a result of chemical modification (cf.(2)). The thiol reagent DTNB* causes the release of a light chain without affecting the Ca^{2+}-ATPase activity of myosin (3,4).

On the basis of fluorescence measurements, we have recently shown that the DTNB light chain from rabbit myosin binds calcium ions, while the other light chains do not. Two dissociation constants were evaluated, one of which is in the micromolar range (5). Ca^{2+} ions were found to affect also the electrophoretic mobility of the DTNB light chains only (6).

Since the stimulation of muscle takes place also in the micromolar range, we looked for a possible relationship between the calcium sensitivity of actomyosin and the presence of the DTNB light chains. The functional significance of these light chains has hitherto been unknown and we hoped that this study could clarify their role. For this purpose we measured the calcium sensitivity of complexes formed between DTNB-treated myosin or HMM with actin, in the presence of the regulatory system of the thin filaments.

*Abbreviations: DTNB, 5,5'-dithio-bis-2-nitrobenzoic acid; HMM, heavy meromyosin; DTT, dithiothreitol.

Materials and Methods: Myosin was extracted from the back muscles of rabbits as previously described (7). Heavy meromyosin (HMM) was obtained from myosin (8) by a 10 min digestion at neutral pH at room temperature and was further purified by ammonium sulfate fractionation (9). Both materials were stored at -18° in 50% glycerol, which was removed before use by dialysis. Actin was prepared from acetone dried powder of muscle (10) except that the buffer used was Tris. Troponin-containing tropomyosin was prepared from the same powder (11). DTNB was a Sigma product.

Preparation of DTNB-treated myosin (DTNB myosin): Myosin (10 mg/ml was incubated in the cold (0-4°) with $1x10^{-2}$M DTNB in the presence of $1x10^{-2}$M EDTA, $4.3x10^{-2}$M Tris buffer pH 8.5 and 0.5M KCl (3). After 10-15 min the solution was diluted with 10 volumes of cold water and the precipitate was collected by centrifugation at 9,000 rpm (15 min). The precipitate (containing DTNB-myosin) was dissolved in KCl (0.5M), dithiothreitol (DTT) added up to 2 mM, and the solution dialyzed exhaustively (against DTT solutions containing $1x10^{-2}$M EDTA). After the unblocking of -SH groups was completed, i.e. when the dialyzate was no longer yellow, the DTNB-myosin solution was further dialyzed against 0.5 M KCl -- 0.05 M Tris buffer pH 7.4, in order to remove both the DTT and EDTA. This solution was then centrifuged at 20,000 rpm for 1.5 h, to get rid of aggregates. This solution could be stored in 50% glycerol. DTNB-HMM was obtained from DTNB-myosin in the same manner as control HMM (see above). There was absolutely no difference between the UV spectrum of DTNB-HMM and that of HMM, thus indicating that all the -SH blocking groups had been removed.

Protein concentrations were determined either by the biuret method or by UV absorption ($E^{1\%}_{1cm}$ at 278 nm: HMM-6.33; myosin-6.35). Enzyme activity was measured with a Radiometer titrigraph TTT1 at pH 7.5, 25°. The assays were: Ca^{2+} or Mg^{2+}-ATPase -- 4mM Ca^{2+} or Mg^{2+}, 2.5 mM ATP; 50 mM KCl; 0.2-0.8 mg myosin; actin-activated ATPase -- same as for Mg^{2+}-ATPase, with actin: tropomyosin:myosin = 1:1:0.6-1, the KCl con-

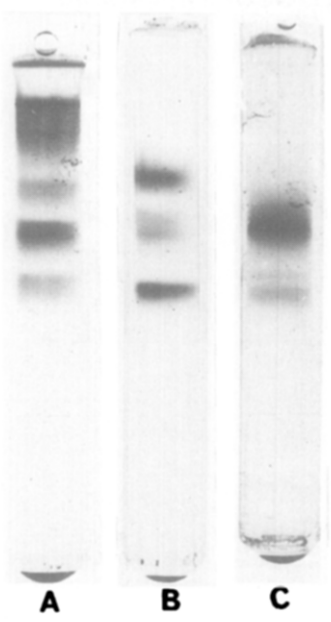

Figure 1: Analytical disc-gel electrophoresis pattern of light chains in 6% acrylamide gels containing 6M urea obtained from: A. Myosin; B. DTNB-myosin; C. Light chains released from myosin by DTNB treatment.

centration varying between 25 and 100 mM; EDTA-activated ATPase -- 1 mM EDTA, 2.5 mM ATP, 500 mM KCl, 0.2-0.4 mg myosin. The volume of the assay solution was 3 ml and 0.01 or 0.02 N NaOH was used as titrant. The slopes of the straight lines obtained during the first few minutes of the reaction were taken as the steady state rate of ATP hydrolysis. The rates were corrected for the incomplete dissociation of phosphate-pKa 7.2 (12) at pH 7.5. When Ca^{2+} sensitivity was measured the assay medium was the same as that for actin-activated ATPase, and Ca^{2+}-EGTA buffer solutions were added. The free Ca^{2+} concentrations at pH 7.4 were computed using a value of 1.86×10^{7} M^{-1} for the apparent dissociation constant of Ca^{2+}-EGTA (calculated from "Stability Constants" data (13)). Fluorescence measurements were performed as previously described (14). Analytical gel electrophoresis was performed in 6% polyacrylamide gels containing 6 M urea (15).

Results: Treatment of myosin with DTNB, followed by removal of the blocking group with DTT, caused the release of the light chains of intermediate

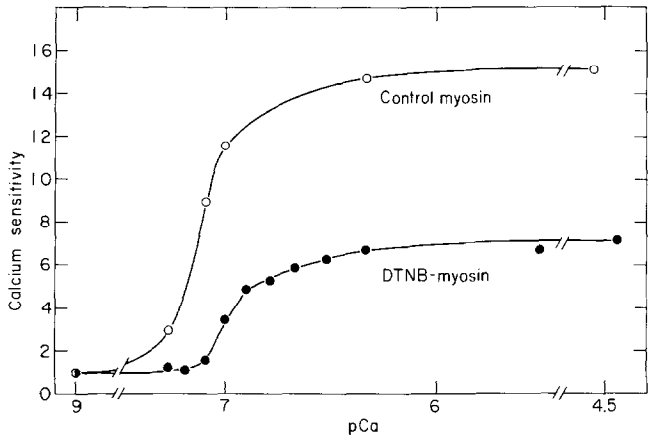

Figure 2: Calcium sensitivity of acto-DTNB-myosin as compared to control actomyosin, using EGTA-Ca^{2+} buffers. 0.56 mg myosin or DTNB-myosin, 0.88 mg actin, 0.82 mg troponin-containing tropomyosin, 4 mM Mg^{2+}, 2.5 mM ATP, 53 mM KCl, 187 µM EGTA and Ca^{2+} varying from 0 to 225 µM.

mobility (LMP-II (15)) (Fig. 1). This "DTNB-myosin" had lost about 55% of its LMP-II as measured by densitometry of urea-polyacrylamide gels of the remaining light chains. However, this is probably a low limit, since LMP-II, which is the predominant band in control myosin, becomes the faintest one in DTNB-myosin.

Although the Ca^{2+}- and Mg^{2+}-ATPase activities of DTNB-myosin are practically unaffected, both its EDTA-ATPase and actin-activated ATPase activities are greatly reduced (Tables I and II). The last activity is also less dependent on ionic strength than that of control myosin (Table II).

When the actin-activated ATPase of DTNB-myosin was measured in the presence of troponin and tropomyosin, a considerable decrease in the Ca^{2+} sensitivity of the system was observed. As can be seen from Fig. 2, the maximal reduction (about 5-fold) occurs at $[Ca] \simeq 7 \times 10^{-8}$ M whereas above 10^{-6} M the ratio of the Ca^{2+} sensitivities of control and DTNB myosins is about 2.

The actin-activated ATPase activity of DTNB-HMM prepared from DTNB-myosin was also drastically reduced, although its Ca^{2+}-ATPase was only slightly affected (80% of control). Double reciprocal plots of acto-DTNB-HMM ATPase against actin concentration (16) show that V_{max} was reduced to

TABLE I

Ca^{2+}- and EDTA-ATPase activities of DTNB- and control-myosins (in μmole/min/mg)

	Ca^{2+}ATPase	Mg^{2+}ATPase	EDTA-ATPase
Control myosin	0.46 ; 0.53	0.036	1.39 ; 1.82
DTNB-myosin	0.43 ; 0.52	0.031	0.63 ; 0.57

18% of the control, while the apparent dissociation constant was 5-fold lower than that of control acto-HMM.

There was practically no change in the fluorescence of DTNB-HMM upon removal or addition of Ca^{2+} or Mg^{2+} ions (Table III). On the other hand, the effect of these metal ions on the fluorescence of the light chain preparation, obtained by treating myosin with DTNB, corresponded to 80% of that of pure LMP-II (5).

Discussion: The fact that DTNB treatment of myosin causes a decrease in EDTA ATPase activity is characteristic of the action of sulfhydryl reagents (17). The lower actin activation found appears to be associated with the decrease in EDTA activation (18). A reduction in ionic strength sensitivity of the actin-activation of DTNB-myosin (Table II) was previously observed in the case of DTNB-treated natural actomyosin (19). It is interesting to note that an increase in the apparent binding constant to F-actin was also reported for pCMB-modified myosin (20).

Our measurements clearly show that DTNB treatment of myosin impairs the calcium sensitivity of actomyosin. In principle DTNB, in addition to releasing light chains, might affect other parts of the myosin molecule. However, the following arguments can be brought forward in favor of the

TABLE II

Actin-activated ATPase activity of DTNB- and control myosins
(in μmole/min/mg myosin)

[KCl] (mM)	25	53	100
Control myosin	1.01	0.690	0.089
DTNB-myosin	0.30	0.276	0.066

Assay: 0.56 mg myosin or DTNB-myosin, 0.88 mg actin, 0.82 mg troponin-containing tropomyosin.

idea that the DTNB light chains are required for the calcium sensitivity of natural actomyosin:

a) The intimate relationship between the presence of these chains and calcium binding of myosin. It has been reported (21) that myosin takes two calcium ions from a 10^{-6}M solution. As has previously been shown, the intrinsic fluorescence of myosin and of HMM is affected by these ions (22). This effect disappears when the calcium-binding DTNB light chains are missing from HMM (Table III).

b) The other light chains ("alkali chains") do not appear to be involved in Ca^{2+} binding. Thus, after the removal of these chains, myosin still binds 2 calcium ions (23) and negligible changes are induced by Ca^{2+} in the fluorescence or in the electrophoretic mobility of the alkali chains (5,6).

c) The fact that DTNB light chains can bind calcium ions in the same concentration range as that required for the stimulation of actomyosin.

d) The finding that the loss of calcium sensitivity takes place concurrently with removal of the DTNB light chains.

We may therefore conclude that the DTNB light chains have a regulatory role in the thick filaments of skeletal muscle (5,6) similar to that of

TABLE III

Effect of EDTA on the fluorescence of HMM, DTNB-HMM and DTNB-released light chains

Protein	$\Delta I/I$[a]	Relative effect
HMM	6.6	1.00
DTNB-HMM	0.6	0.09
Pure LMP-II	21.1	1.00
DTNB-released light chains	16.9	0.80

[a] $\Delta I/I$ is the difference between the fluorescence intensity in the presence of EDTA and the intensity I in the presence of saturating concentrations of Ca^{2+} or Mg^{2+}.

troponin in the thin filaments. Actually, it is quite "logical" that sensitization should involve the thick filaments, which are the only ones that are capable of sending "arms" towards the other set of filaments and interact with them the moment the muscle is stimulated. The fact that the ATPase activity of synthetic actomyosin is not calcium sensitive even though the DTNB light chains are present, suggests that, in skeletal muscle, regulation is a process involving the DTNB light chains together with troponin and tropomyosin.

This work was supported by a grant from the Muscular Dystrophy Association of America.

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