A COMPARATIVE STUDY OF MUSCLE CONTRACTION AND SUPERPRECIPITATION USING TRINITROPHENYLATED MYOSIN AND ACTIVE MYOSIN SUBFRAGMENTS

Hanna Gadasi, Avraham Oplatka, Raphael Lamed and Andras Muhlrad

Department of Polymer Research, The Weizmann Institute of Science, Rehovot, Israel

:ceived April 8,1974

MMARY: Myosin and heavy meromyosin (HMM) were trinitrophenylated (TNP-ed) to different tents by 2,4,6-trinitro benzene sulfonate. Protein preparations in which only one active te per molecule has been TNP-ed were isolated by using immobilized ATP affinity chromato-aphy columns. Irrigation of "ghost" myofibrils (i.e. myofibrils from which the myosin s been extracted) with either of these "one-headed" species, followed by the addition of ATP, caused contraction. The myosin derivative exhibited also superprecipitation. On the her hand, fully TNP-ed myosin and HMM could not induce contraction of ghosts and the rst did not give superprecipitation. The relationship between superprecipitation and scle contraction is discussed.

In another communication (submitted to Biochem.Biophys.Res.Comm.) we have shown that ofibrils from which myosin has been extracted ("ghost" myofibrils), irrigated with HMM S-1 -1), contracted upon adding MgATP. In order to further confirm our conclusion that a osin "head" can generate a mechanochemical force irrespective of whether or not its twin ad is present, we have now prepared other "one-headed" myosin species and tested them for eir capability to induce contraction of ghost myofibrils: Myosin which has been tri-trophenylated (TNP-ed) with trinitro benzene sulfonate (TBS) at both its active sites is rongly activated by Mg^{2+}, the maximal activity not being enhanced by actin (1). Ghost brils which have been irrigated with fully TNP-ed myosin (or HMM) do not contract upon ding MgATP, while irrigation with the native proteins "reconstituted" the ghosts into ntractile elements (2). We therefore partly TNP-ed myosin and HMM and, using immobilized P columns [(3); also Lamed and Oplatka, submitted to Biochemistry], isolated fractions ntaining myosin or HMM in which only one active site was TNP-ed.

The phenomenon of "superprecipitation" has been considered as some kind of a model r muscle contraction. In order to learn whether the concerted action of myosin's two ads is required for contraction, the superprecipitation of myosin which has partially en inactivated or lost one of its heads by the action of a proteolytic enzyme were in-stigated by two groups of investigators (4,5,6) who arrived at contradictory conclusions. therefore checked one-headed TNP-myosin for superprecipitation.

TERIALS AND METHODS

Myosin and actin were prepared (7,8) using the white back muscles of New Zealand ite rabbits. HMM, obtained from myosin (9), was further purified by passing it through Sepharose adipic dihydrazide-ATP (Seph-ADH-ATP) column (3): About 100 mg protein in)-40 ml were applied to a 3.5x15 cm column after dialyzing against a solution containing) mM KCl, 10 mM imidazole buffer pH 7.0 and 1 mM EDTA. The inactive protein was washed

out with the equilibrating solution and the active enzyme, which exhibited a single symmetrical peak in a KCl gradient, was eluted with a similar solution at a higher ionic strength (0.8 M KCl). The Ca^{2+}-ATPase of the eluted HMM was about one µmole/min/mg. Myofibrils were prepared from a mixture of rabbit back and psoas muscles (10), kept in 50% glycerol at -18°C and suspended in a "standard salt solution" (s.s.s.) containing 0.1M KCl, 1 mM $MgCl_2$ and 20 mM phosphate buffer pH 7.0. ATPase activity, was measured essentially by a modified Fiske and SubbaRow's method (11). Conditions: 12.5-25 µg protein per ml, 1 mM ATP, 20 mM Tris-HCl pH 8.0 and either 5 mM EDTA - 0.6 M KCl or 2 mM $MgCl_2$ - 0.4 M KCl, 25°. Activities are expressed as µmoles P_i/min/mg enzyme.

Preparation of "one-headed" TNP-myosin: Myosin was TNP-ed by TBS (TBS: myosin = 5.1) (1). The degree of TNP-ation was calculated from the change in absorbance at 345 nm (12). 40 mg protein, containing 1.2 moles TNP per mole myosin were loaded on a 2.0x26 cm Sepharose-Sebacic acid-dihydrazide-ATP (Seph-SEH-ATP) column previously washed with a 0.4 M KCl, 4 mM $MgCl_2$, 0.5 mM EDTA, 10 mM borate pH 8.0 solution. 5 ml fractions were collected by washing with 70 ml of the equilibrating solution, followed by 200 ml of a linear KCl gradient. Finally, 135 ml of a linear pyrophosphate gradient, containing 1 M KCl, was employed. The EDTA- and Mg^{2+}-activated ATPases and the protein content of the fractions were determined [Fig. 1(A)]. We have recently found (Muhlrad, Lamed and Oplatka, in preparation) that, while myosin is completely eluted by a 0.4-3 M KCl gradient, myosin "labelled" at both active sites cannot be eluted by the same KCl gradient but it can be extracted with a 0-0.1 M pyrophosphate gradient. Myosin is thus confined to the KCl-eluted peak while the second eluted peak contains the "fully" TNP-ed myosin. One-headed TNP-myosin should thus be distributed between these two peaks. It is known (1) that upon TNP-ing myosin, the EDTA-ATPase is diminished while the Mg^{2+}-ATPase is enhanced. If we denote the corresponding specific activities of native myosin by a and b, and those of the fully TNP-ed myosin by c and d, then the ratio of the activities for a given fraction is equal to $[a(100-x)+c.x]\cdot[b(100-x)+d.x]^{-1}$ where x is the percentage of the TNP-ed active sites. With: a=1.8, b=0.005, c=0.05 and d=0.12, the concentrations of the three myosin species could be calculated [Fig. 1(b)], assuming the specific activity of the one-headed species to be the average of the other two. The pooled myosin fractions 61-64 were taken as pure one-headed TNP-myosin, after concentration by dry Sephadex C150; its Ca^{2+}, EDTA- and Mg^{2+} activities were 0.22, 0.68 and 0.07 respectively.

Preparation of "one-headed" TNP-HMM: TBS was added to HMM in a molar ratio of 3.6:1 (1). Excess TBS was removed by dialysis against 100 volumes of 30 mM KCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 10 mM imidazole pH 7, with two changes, for 24 hours. 1.14 moles of TNP per mole HMM were found to be incorporated. A 0.8x16 cm Seph-ADH-ATP column, previously washed with an equilibrium buffer (30 mM KCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 10 mM imidazole pH 7.0) was loaded with 16 mg protein. 2 ml fractions were collected by washing with 48 ml equilibrium buffer, followed by 120 ml of a linear KCl

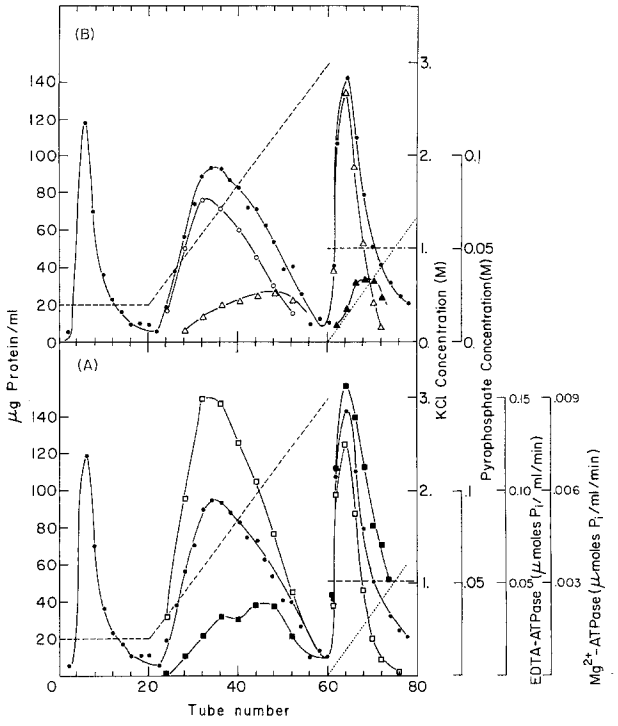

Fig. 1: Purification of "one-headed" TNP-myosin on a Seph-SEH-ATP column: ●-● total protein (μg/ml). --- KCl concentration. ..... sodium pyrophosphate concentration.
Bottom (A): ◘-◘ EDTA-ATPase; ■ - ■ Mg^{2+}-ATPase.
Top (B) (Protein content in μg/ml): o-o normal myosin; Δ-Δ one-headed TNP-myosin; ▲-▲ fully TNP-ed myosin.

gradient. The protein content and the EDTA- and Mg^{2+} activities were determined [Fig.2(A)]. The concentration in the eluted fractions of unmodified HMM and of HMM TNP-ed in one and in two active sites were evaluated as for TNP-myosin [Fig.2(B)]. The specific activities were: a=1.6, b=0.004, c=0.06 and d=0.128. The protein in fractions 60-64 contains practically pure one-headed TNP-HMM; its EDTA- and Mg^{2+} activities were 0.83 and 0.074.

Trinitrophenylation of S-1: TBS was added to S-1 in a molar ratio of 10:1 (1). Excess TBS was removed as for TNP-HMM. The degree of TNP-ation was 2.2-2.4 moles/mole S-1, indicating TNP-ion of other parts of the molecule, in addition to the active site. The Ca^{2+}- and actin-activities were 0.092, 0.05 and 0.05, respectively.

Contraction of irrigated "ghost" fibrils: Myosin was extracted from myofibrils under a phase-contrast microscope (magnification 1,250-3,000) by a Hasselbach-Schneider solution and the ghosts then washed with s.s.s. (see MATERIALS AND METHODS) (13). [Addition of MgATP at this stage did not cause any contraction.] This was followed by

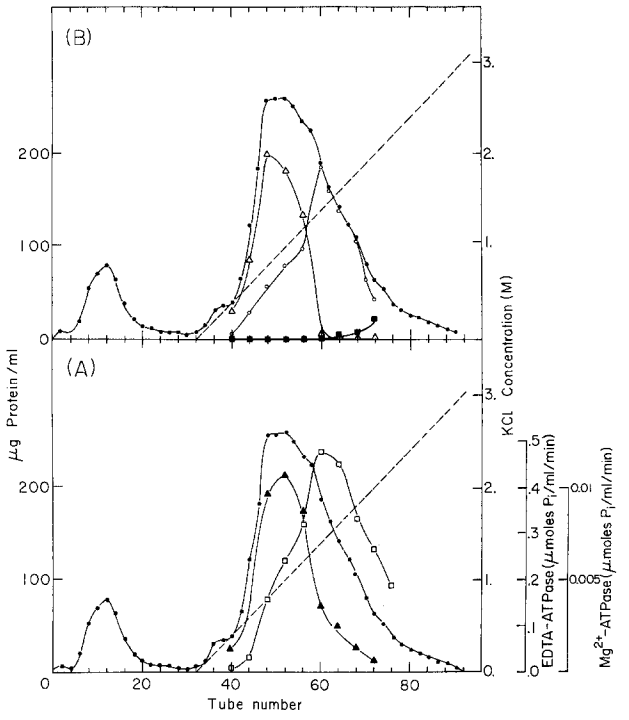

Fig. 2: Purification of "one-headed" TNP-HMM on a Seph-ADH-ATP column: ●-● total protein (μg/ml); --- KCl concentration. Bottom (A): ▲-▲ EDTA-ATPase; ◘ - ◘ Mg^{2+}-ATPase. Top (B): (Protein content, in μg/ml): Δ-Δ HMM; o-o "one-headed" TNP-HMM; ■ - ■ fully TNP-ed HMM.

the addition of a few small drops of either of the one-headed TNP species (2 mg/ml, 5 mM Tris buffer pH 7.6 and either 0.4 M KCl, for the modified myosin, or 50 mM KCl for the HMM derivative). In the case of TNP-myosin, the ionic strength was lowered by washing with s.s.s. prior to the application of the contracting solution (1 mM MgATP + 2 mg/ml protein).

RESULTS AND DISCUSSION

One-headed TNP-myosin reconstituted fibrils contracted in practically all experiments while in the case of one-headed TNP-HMM contraction occurred in about 70% of the experiments (about 100 for each protein). This is in line with our previous observation that irrigation of ghost myofibrils with S-1 caused contraction upon the addition of MgATP.

Irrigation with TNP-S-1 made the ghosts appear darker. The addition of MgATP made them clear again, probably due to dissociation of complexes formed with actin; however, no contraction occurred, similarly to irrigation with fully TNP-ed myosin or HMM (2).

Taking these results together with the fact that actin does not increase the maximal Mg^{2+}-activated ATPase activity of TNP-ed myosin, it seems that TNP-ation of myosin's active sites causes the uncoupling of mechanochemical transduction by simply preventing the formation of actin-myosin "active" complexes

One-headed TNP-myosin was found to undergo superprecipitation (Fig.3), similarly

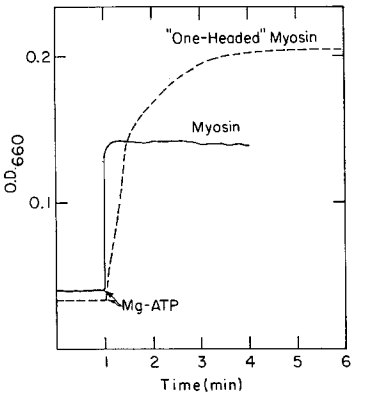

Fig. 3: Superprecipitation of "one-headed" TNP-myosin:
The reaction mixture contained 0.14 mg/ml myosin or "one-headed" TNP-myosin, 0.07 mg/ml actin, 80 mM KCl, 50 mM Tris-acetate buffer pH 7.4 and 0.16 mM Mg-ATP. The process was followed (14) by recording the turbidity at 660 nm.

to Lowey and Margossian's one-headed myosin (5,6). The onset of superprecipitation occurred at the same time, and the rate did not differ much for TNP-ed and native myosin. This is not surprising if one recalls that the activity of fully TNP-ed myosin in the presence of Mg^{2+} is practically equal to the actin-activated ATPase of native myosin (1). The extent of superprecipitation appeared, however, to be markedly higher in the case of the TNP-species. Since fully TNP-ed myosin at low Mg^{2+} concentration does not exhibit superprecipitation (15), it appears that active complex formation is a prerequisite for superpreciptitation, in addition to "rigor" complex formation which takes place at the low ATP concentration prevailing during the process. From viscosity measurements, fully TNP-ed myosin has been found to form a complex with actin at a high ionic strength (2). Upon adding MgATP viscosity dropped and, after a while, increase again. This is reminiscent of the behavior of ghost fibrils irrigated with TNP-S-1. TNP-ed, in addition to native, active sites can thus participate in rigor complex formation during superprecipitation. Since one-headed TNP-myosin can induce the contraction of ghost myofibrils (through its native head) and can form rigor complexes (through its native and chemically-modified heads) there is no a priori reason why it should be able to superprecipitate. It is probable that the tension

generated by the active complexes re-aligns the network formed so as to make it denser. The fact that the extent of superprecipitation in the case of one-headed TNP-myosin exceeds that of native myosin may then suggest that the actin filaments are more flexibl in the presence of TNP-ed myosin. [cf. (16)]. We have recently demonstrated (2,17,18) that actomyosin (or acto-HMM) solutions, confined in glass microcapillaries in the presence of ATP, exhibit vigorous active streaming, reminiscent of cytoplasmic streaming. The velocity of streaming becomes much slower late in the process when superprecipitatio starts. The "clearing-phase" which precedes superprecipitation is thus probably a better representative of contraction than superprecipitation which has the characteristics of both rigor and contraction. One-headed TNP-HMM, similarly to HMM and to S-1, cannot superprecipitate even though they can induce the contraction of ghost fibrils as well as active streaming (2). All this suggests that the absence of superprecipitation should not be taken as an indication for the non-existence of mechanochemical transformation without which, after all, muscle contraction cannot occur.

Supported by grants from the Muscular Dystrophy Associations of America.

REFERENCES

1. Fabian, F. and Muhlrad, A. (1968) Biochim.Biophys.Acta 162, 596.
2. Oplatka, A., Gadasi, H., Tirosh, R., Lamed, Y., Muhlrad, A. and Liron, N. (1974) J.Mechanochem.Cell Motility 2, in press.
3. Lamed, R., Levin, Y. and Oplatka, A. (1973). Biochim.Biophys.Acta 305, 163.
4. Tokiwa, T. and Morales, M.F. (1971) Biochemistry 10, 1722.
5. Margossian, S.S. and Lowey, S. (1973), J.Mol.Biol. 74, 313.
6. Lowey, S. and Margossian, S.S.(1974), J.Mechanochem.Cell Motility 2, in press.
7. Azuma, N. and Watanabe, S. (1965), J.Biol.Chem. 240, 3847.
8. Lehrer, S.S. and Kerwar, G. (1972), Biochemistry 11, 1211.
9. Tokuyama, H., Kubo, S. and Tonomura, Y. (1969), Biochem.Z. 345, 57.
10. Bendall, J.R. (1961), Biochem.J. 81, 520.
11. Bárány, M., Conover, T.E., Schliesefeld, H., Gaetjens, E. and Goffart, M. (1967), Eur.J.Biochem. 2, 156.
12. Okuyama, T. and Satake, K. (1960), J.Biochem. 47, 454.
13. Hanson, J. and Huxley, H.E. (1955), Symp.Soc.Expt.Biol. 9, 228.
14. Ebashi, S. (1961), J.Biochem. 50, 236.
15. Azuma, N. and Watanabe, S. (1973), J.Biochem. 73, 183.
16. Gadasi, H., Oplatka, A., Lamed, R., Hochberg, A. and Low, W. (1974), Biochim.Biophys.Acta 333, 161.
17. Oplatka, A. and Tirosh, R. (1973), Biochim.Biophys.Acta 305, 684.
18. Tirosh, R., Oplatka, A. and Chet, I. (1973) FEBS Letters 34, 40.