THYROID MYOSIN FILAMENT ASSEMBLY–DISASSEMBLY IS CONTROLLED BY MYOSIN LIGHT CHAIN PHOSPHORYLATION–DEPHOSPHORYLATION

François MARTIN*, Jacqueline GABRION and Jean-Claude CAVADORE
Centre de Recherches de Biochimie Macromoléculaire du CNRS, U-249 INSERM and Faculté de Médecine, Montpellier, France

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1. Introduction

Considerable effort has been devoted to the understanding of the molecular basis of non-muslce cell motility and cytoplasmic movements and of their regulations. Actin, myosin, calmodulin, myosin light chain kinase were found to be present in a variety of non-muscle tissues and cells and thought to be involved in the control by Ca^{2+} and/or cyclic AMP of such contractile processes that occur e.g. during mitosis, secretion or endocytosis (review [1,2]).

Smooth muscle contraction is widely believed to be triggered by an increase of cytosolic [Ca^{2+}] through Ca^{2+} binding to calmodulin and subsequent formation of the active ternary complex Ca^{2+}–calmodulin–myosin light chain kinase [3–5]. Phosphomyosin Mg^{2+}-ATPase activity is enhanced by actin and contraction occurs.

Under physiological concentrations of KCl and Mg^{2+}-ATP, gizzard dephosphomyosin was found to be mostly in a dimeric form [6]. In contrast, phosphomyosin is able to form bipolar filaments. Similar observations were reported [7] using non-muscle myosins from thymus and platelets, suggesting that light chain phosphorylation–dephosphorylation might be a general mechanism in the regulation of non-muscle myosin assembly–disassembly.

As a first step towards the understanding of cell motility regulatory pathways, we undertook the isolation of hog thyroid myosin and the study of its ATPase activity and assembly state under physiological conditions when either dephosphorylated or fully phosphorylated.

Abbreviations: MLCK, myosin light chain kinase; EGTA, ethylene glycol bis (β-aminoethyl ether *N,N'*-tetraacetate; DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; SDS, sodium dodecyl sulfate

* To whom correspondence and reprint requests should be addressed at CRBM-CNRS, BP 5051, 34033-Montpellier, France

2. Materials and methods

2.1. *Materials*

Hog thyroid myosin was purified to >98% homogeneity from fresh glands obtained from the local slaughter house as described elsewhere (F. M., in preparation). The specific K^+,EDTA-ATPase and Mg^{2+}-ATPase activities in the presence of 0.6 M KCl at 37°C were 500 nmol/(mg . min) and 19 nmol/(mg . min), respectively. A single type of alkali-light chain M_r 17 000 and the phosphorylatable light chains M_r 20 000 are present.

Calmodulin was prepared from ram testis as in [8]. Actin was isolated from rabbit skeletal muscle according to [9]. Calmodulin-dependent MLCK was prepared either from turkey gizzard [5] or from canine skeletal muscle (A. Molla, J. G. Demaille, J.-C. C., submitted). Both enzymes were strictly Ca^{2+}- and calmodulin-dependent and exhibited spec. act. 2.4 and 4.0 μmol/(min . mg) for the smooth and skeletal enzymes, respectively.

Phosphoprotein-phosphatase S was partially purified according to [10], through the ammonium sulfate and ethanol precipitation steps to spec. act. 0.5 nmol phosphate released/min from phosphohistone mixture. Partially purified enzyme was stored in 1 mM DTT, 1 mM $MnCl_2$ and 50 mM Hepes buffer (pH 7.4). ATP was purchased from Boehringer and [γ-^{32}P]ATP, 1000 Ci/mmol, was from Amersham. Homogeneity of protein preparations was checked by SDS–polyacrylamide gel electrophoresis [11]. Protein concentration was determined by using the Coomassie blue technique [12], with γ-globulin as standard.

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2.2. *Myosin phosphorylation–dephosphorylation*

Myosin (0.6–0.8 mg/ml 0.6 M KCl, 10 mM $MgCl_2$, 1 mM EGTA, 0.1 mM DTT, 20 mM Tris buffer, pH 7) was dephosphorylated in the presence of 20 μg protein phosphatase/ml for 30 min at 30°C. Myosin was then freed of phosphatase by gel filtration on a Sepharose 6B column (0.9 × 85 cm) equilibrated with the above buffer and eluted at 3 ml/h.

Before phosphorylation, the myosin solution (0.8 mg/ml) was dialyzed against 0.1 M KCl, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM DTT, 20 mM Tris–HCl buffer (pH 7.5). Calmodulin (0.5 μM) and MLCK (0.1 mg/ml) were then added together with 0.5 mM [γ-^{32}P]ATP (150 cpm/pmol). After 30 min incubation at 30°C, myosin was freed of calmodulin and MLCK by gel filtration as above.

The extent of phosphorylation was measured at various times by withdrawing 20 μl aliquots that were added to 0.1 ml 10 mM ATP, 10 mg bovine serum albumin/ml. Proteins were immediately precipitated at 0°C by addition of 1 ml 2 mM ATP, 15% (w/v) trichloroacetic acid, left on ice for 10 min and spun down in a clinical centrifuge. The pellet was redissolved at 0°C with 0.1 ml 0.2 N NaOH and precipitated again by addition of 1 ml ATP–trichloroacetic acid mixture, and this procedure was repeated twice. Finally, the pellet was dissolved in 0.5 ml 98% formic acid and counted in dioxane–naphthalene scintillant.

2.3. *ATPase activity*

Actomyosin Mg^{2+} ATPase activity was measured in 0.1 mM $CaCl_2$, 10 mM $MgCl_2$, 60 mM KCl, 2.5 mM ATP, 10 mM imidazole buffer (pH 7.0) in the presence of actin and myosin (1.5 and 0.1 mg/ml, respectively). After 30 min incubation at 37°C, the reaction was quenched by addition of trichloroacetic acid to 5% final conc. and orthophosphate was measured as in [13].

2.4. *Electron microscopy*

Dephospho- or phosphomyosin solutions (0.15 mg/ml) were dialyzed overnight *vs* 0.15 M KCl, 10 mM $MgCl_2$, 1 mM EGTA, 0.1 mM DTT, 25 mM imidazole buffer (pH 7.0) and were eventually adjusted to various ATP concentrations. The solution (10 μl) was pipetted onto a 200 mesh Formvar–carbon-coated grid. After 1–2 min, the grid was washed with 10 drops of the above buffer, then negatively stained with 10 drops of 0.5% (w/v) uranyl acetate; grids were air dried and examined by using a Philips EM 200 electron microscope operating at 80 kV with a 20 μm aperture.

3. Results

3.1. *Myosin phosphorylation–dephosphorylation*

When phosphorylated by MLCK in the presence of [γ-^{32}P]ATP and examined by SDS–polyacrylamide gel electrophoresis, only the 20 000 M_r regulatory light chain was found to be phosphorylated. Incorporation of [^{32}P]phosphate with MLCK was used as an indirect measurement of the remaining non-radioactive phosphate. As shown in fig.1, freshly isolated myosin appeared to contain ~1 mol phosphate/mol since 0.9 mol ^{32}P/mol could be incorporated after incubation with the kinase. In contrast, phosphatase-treated myosin was found to be essentially free of phosphate, since 1.9 mol ^{32}P could be incorporated/mol. Equivalent results were obtained by using either skeletal or gizzard MLCK.

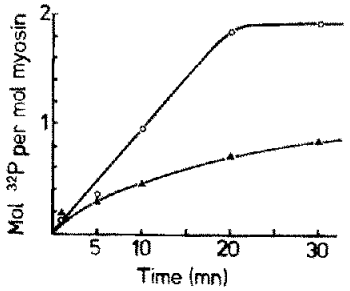

Fig.1. Time course and extent of ^{32}P incorporation into dephosphorylated (○) or untreated (▲) thyroid myosin in the presence of myosin light chain kinase. Conditions were as in section 2.

Table 1
Mg^{2+}-ATPase activity of hog thyroid myosin

	Phosphatase-treated myosin	Untreated myosin	Phosphorylated myosin
Phosphatase content (mol/mol)	0.1	1.1	2
Mg^{2+}-ATPase activity (nmol P_i/(mg . min))			
− actin	5	12	4.5
+ actin	7	43	144

Table 1 lists the values of Mg^{2+}-ATPase activities of myosin preparations with various extents of phosphorylation. Dephosphomyosin ATPase activity is not sensitive to addition of actin which enhances 20-fold the activity of fully phosphorylated myosin.

3.2. *Myosin assembly–disassembly*

In the presence of 10 mM Mg^{2+} and 1 mM EGTA and in the absence of ATP, untreated myosin formed bipolar filaments with a 375 ± 15 nm length, 16 nm diameter and a bare zone of 167 ± 12 nm, as shown in fig.2a,b. When dephosphorylated myosin was used, bipolar filaments were also obtained under the same conditions; therefore they appeared shorter (as short as 250 nm) than those observed on untreated myosin.

Upon addition of 5 mM ATP to untreated myosin (fig.2c) or dephosphorylated myosin, the bipolar filaments disappear, leaving only a few rod assemblies without visible heads, presumably generated by proteolysis and insensitive to addition of ATP.

Upon treatment of untreated or dephosphorylated myosin for 15 min at pH 7.0 by 10 mM $MgCl_2$, 1 mM EGTA, 5 mM ATP, 0.5 μM calmodulin and MLCK (150 μg/ml), only a few rod assemblies were observed, as already discussed above. Addition of 1.2 mM $CaCl_2$, i.e., 0.2 mM excess over EGTA, triggered the formation of bipolar filaments within 15 min (fig.2d). Filaments appear tapered and longer (up to 500 nm).

A suspension of phosphomyosin filaments in 5 mM ATP was incubated for 20 min in the presence of excess protein-phosphatase S (100 μg/ml). Dephosphorylation results in complete disappearance of filaments (fig.2e).

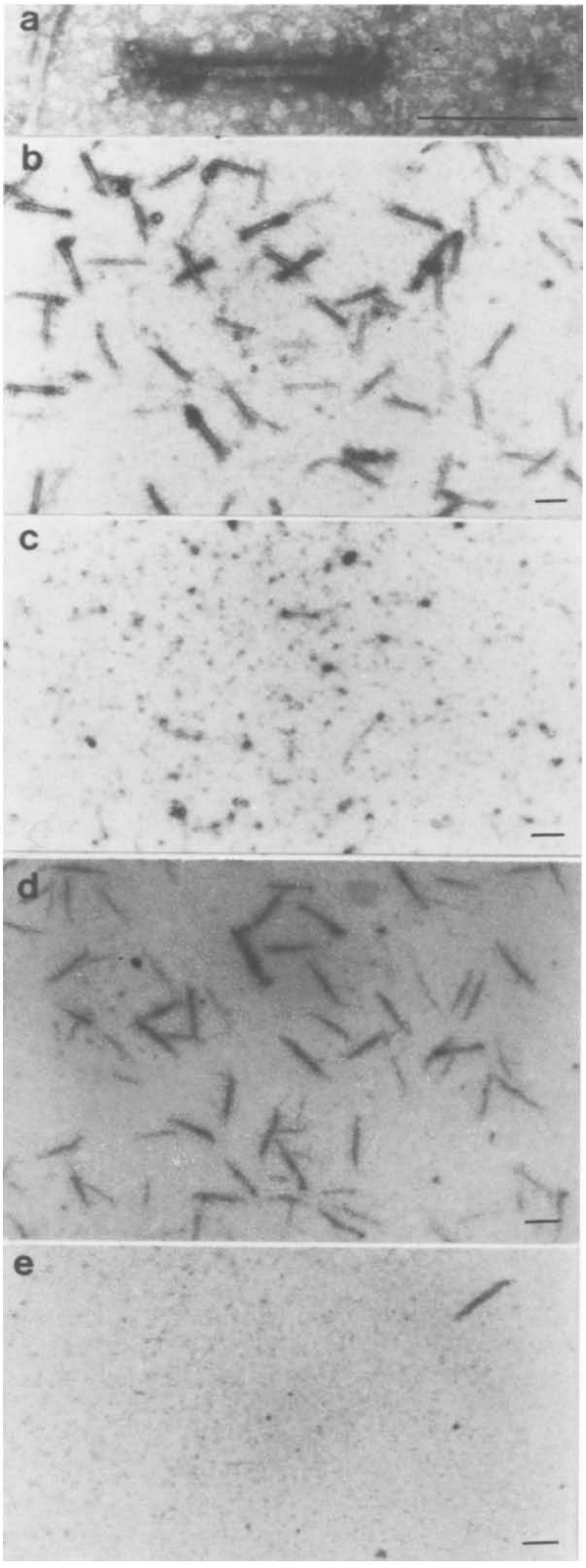

Fig.2. Electron micrographs of hog thyroid myosin, bar = 250 nm: (a,b) untreated myosin, in the presence of Mg^{2+} and EGTA and absence of ATP. The molecules are organized into bipolar synthetic filaments with a clearly visible bare zone: (c) same preparations as in (a,b) after addition of 5 mM Mg ATP; most myosin filaments were dissociated, only a few rods remain visible; (d) same preparation as in (c) after addition of MLCK, calmodulin and Ca^{2+} (conditions in section 2). Myosin reorganized into filaments which appear longer and more spindle-shaped than in (a,b); (e) dephosphorylated myosin: myosin phosphorylated as in (d) was freed of kinase and Ca^{2+} and treated by protein phosphatase as in section 2. In the presence of 5 mM Mg^{2+}-ATP, no myosin filament was visible.

4. Discussion

There is little doubt at this time that phosphomyosin Mg^{2+}-ATPase activity is enhanced by addition of actin whereas dephosphomyosin activity is not. Among the possible explanations of this difference, the assembly of phosphomyosin in filaments has recently received considerable attention [6,7,14–16].

This report strengthens this view and extends it to another non-muscle cell system, the thyroid gland. Two main questions are to be answered in this respect:

(i) The physiological relevance of the phenomenon is emphasized by the finding and partial purification of Ca^{2+}–calmodulin-dependent myosin light chain kinase in the thyroid cell (J.-C. C., A. Molla, F. M., unpublished). Also, antibodies elicited against pure gizzard MLCK specifically stain the apical pole of the cell when visualized by indirect immunofluorescence, with roughly the same distribution as actin (J. G., M.-C. Harricane, J.-C. C., unpublished). Concentrations of ATP used in this paper are within the physiological range [17,18].

It is therefore a distinct possibility that in vivo, myosin assembly is controlled by the free Ca^{2+} level through the calmodulin-dependent kinase, disassembly being promoted by the phosphatases that are either permanently active or switched on when Ca^{2+} level drops to basal values. If this were true, one would not expect to find myosin filaments if precautions are not taken to prevent hydrolysis of phosphoprotein esters during processing of the sample, i.e., presence of Mg^{2+}-ATP, Ca^{2+}, calmodulin and kinase before fixation.

(ii) The mechanism by which phosphorylation of regulatory light chains, supposedly located at the hinge region of myosin between S1 and S2 subfragments [19], promotes myosin assembly through rod–rod interaction, is still a matter of speculation. This implies that light chain phosphorylation may result in a global change in the conformation of the molecule.

Whether or not actin binding sites and ATPase catalytic sites in the myosin heads are affected by light chain phosphorylation is still highly controversial.

These experiments provide a mechanism by which Ca^{2+} can promote contractility of the thyroid cell and especially of its apical portion, actively engaged in endocytosis of thyroglobulin following thyrotropin stimulation.

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