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Purification of smooth-muscle myosin free of calmodulin and myosin light-chain kinase

Susceptibility to oxidation

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Smooth-muscle myosin purified as described by Persechini & Hartshorne [(1983) Biochemistry **22**, 470–476] contains trace amounts of calmodulin and myosin light-chain kinase, which can be removed by Ca^{2+}-dependent hydrophobic-interaction chromatography followed by calmodulin–Sepharose affinity chromatography. The resultant column-purified myosin exhibits properties similar to those of the non-purified myosin, e.g. actin activation of the Mg^{2+}-ATPase requires Ca^{2+}/calmodulin-dependent phosphorylation of the two 20 kDa light chains. However, unlike the non-purified myosin, the column-purified myosin undergoes a time-dependent transition to a form which no longer requires phosphorylation for actin activation of the myosin Mg^{2+}-ATPase. This transition is identified as a time-dependent change in conformation of the column-purified myosin from a 10 S to 6 S form and is caused by slow oxidation of the column-purified myosin, since it could be prevented by storage under N_2 and reversed by 5 mM-dithiothreitol.

INTRODUCTION

The regulation of smooth-muscle contraction by Ca^{2+} is believed to involve phosphorylation of myosin catalysed by the Ca^{2+}/calmodulin-dependent myosin light-chain kinase (Adelstein & Eisenberg, 1980; Walsh & Hartshorne, 1982; Walsh, 1985). Considerable attention has been devoted in recent years to an understanding of the molecular mechanism whereby myosin phosphorylation leads to actin activation of the myosin Mg^{2+}-ATPase. Suzuki *et al.* (1978) originally demonstrated that phosphorylation converted myosin from a 10 S component into a 6 S component. It was shown later (Suzuki *et al.*, 1982; Trybus *et al.*, 1982; Craig *et al.*, 1983) that these represent different conformations of monomeric myosin: the 10 S conformer is folded, whereas the 6 S conformer is extended. Ikebe *et al.* (1983*a*) demonstrated a correlation between transition of smooth-muscle myosin from the 10 S to the 6 S form and conversion of myosin from an inactive into an active state. Phosphorylation of myosin appears to induce an important conformational change in the neck region of the molecule (Ikebe & Hartshorne, 1984). Although these studies have been conducted with monomeric myosin, it is likely that similar changes occur in the neck region of myosin molecules assembled into filaments, in which form myosin is thought to exist in both contracting and relaxed smooth muscle (Somlyo *et al.*, 1981).

In addition to phosphorylation, other independent factors have been shown to induce a transition from 10 S to 6 S myosin: increasing ionic strength (Ikebe *et al.*, 1983*a*), high concentration of Mg^{2+} (Ikebe *et al.*, 1983*a*, 1984), removal of ATP (Ikebe *et al.*, 1983*a*), trinitrophenylation (Srivastava *et al.*, 1985) and modification of two thiol groups with *N*-ethylmaleimide (Chandra *et al.*, 1985). However, under physiological conditions of ionic strength, [Mg^{2+}] and [ATP], phosphorylation induces the conformational transition. In the present paper, we report the slow transition of smooth-muscle myosin, purified through phenyl-Sepharose and calmodulin–Sepharose columns, from a form whose actin-activated Mg^{2+}-ATPase activity is phosphorylation-dependent to a form which is phosphorylation-independent. This transition is shown to be due to conversion of the 10 S into the 6 S conformation by oxidation, and is reversible.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP (10–40 Ci/mmol) was purchased from Amersham Corp. (Oakville, Ontario, Canada). Phenyl Sepharose CL-4B and CNBr-activated Sepharose 4B were purchased from Pharmacia (Mississauga, Ontario, Canada), M_r marker proteins from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and papain was from Boehringer Mannheim (Dorval, Quebec, Canada). General laboratory reagents used were analytical grade or better and were purchased from Fisher Scientific (Calgary, Alberta, Canada).

Preparation of proteins

Calmodulin was purified from frozen bovine brains by a modification of the method of Gopalakrishna & Anderson (1982) as described in detail by Walsh *et al.* (1984) and coupled to CNBr-activated Sepharose 4B as previously described (Walsh *et al.*, 1982). The following proteins were purified by previously described methods: chicken gizzard myosin light-chain kinase (Ngai *et al.*, 1984) and tropomyosin (Bretscher, 1984) and rabbit skeletal-muscle actin (Zot & Potter, 1981). Myosin was purified from frozen chicken gizzards as described by Persechini & Hartshorne (1983), and further purified to remove trace amounts of contaminating calmodulin and myosin light-chain kinase as follows. Myosin (120 mg) was dialysed against two changes (2 l each) of 10 mM-

Tris/HCl (pH 7.5)/0.3 M-KCl/0.2 mM-dithiothreitol. $CaCl_2$ was added to a final concentration of 0.1 mM to the dialysed myosin, which was then loaded, at a flow rate of 22 ml/h, on to a column of phenyl-Sepharose CL-4B (1 cm × 40 cm) coupled in series with two calmodulin–Sepharose columns (1 cm × 20 cm each) arranged in parallel. The three columns were equilibrated and eluted with 10 mM-Tris/HCl (pH 7.5)/0.3 M-KCl/0.2 mM-dithiothreitol/0.1 mM-$CaCl_2$. Fractions of volume 4 ml were collected. Purified myosin was recovered in the flow-through fractions (see the Results section). After washing of the columns with equilibration buffer, bound proteins were eluted with 10 mM-Tris/HCl (pH 7.5)/0.3 M-KCl/0.2 mM-dithiothreitol/1 mM-EGTA.

Biochemical methods

Actin-activated Mg^{2+}-ATPase and myosin Ca^{2+}-ATPase activities were measured under conditions described in the corresponding Figure legends. Myosin phosphorylation was quantified as described by Walsh *et al.* (1983). Protein concentrations were determined by the Coomassie Blue dye-binding assay (Spector, 1978) with dye reagent and γ-globulin standard purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.), or by spectrophotometric measurements, by using the following absorption coefficients: calmodulin, $A^{1\%}_{277} = 1.9$ (Klee, 1977); myosin $A^{1\%}_{280} = 4.5$ (Okamoto & Sekine, 1978). Papain digestion of gizzard myosin was carried out essentially as described by Ikebe & Hartshorne (1984). Papain was activated by incubation at 1 mg/ml for 60 min at 35 °C in 50 mM-Tris/HCl (pH 7.5)/0.5 mM-EDTA/20 mM-dithiothreitol. Preliminary experiments indicated that a papain:myosin ratio of 1:500 (w/w) was suitable for generating a limited digest. Myosin (1.0 mg/ml) was digested at 25 °C by activated papain (2 μg/ml) in 30 mM-Tris/HCl (pH 7.5)/0.2 M-KCl/1 mM-ATP. Other methods were as previously described (Clark *et al.*, 1986).

Biophysical methods

Intrinsic tryptophan fluorescence of myosin was measured in a Perkin–Elmer model 650-10M fluorescence spectrophotometer with excitation wavelength 295 nm and emission wavelength 340 nm (Ikebe *et al.*, 1983*b*). Myosin (0.5 mg/ml) was incubated at 22 °C with 30 mM-Tris/HCl (pH 7.5), 10 mM-$MgCl_2$, 0.1 mM-EGTA, 0.1 M- or 0.3 M-KCl, 5 μg of myosin light-chain kinase/ml and 5 μg of calmodulin/ml in a total volume of 3.0 ml. The fluorescence of this solution was measured against distilled deionized water. At $t = 0$, ATP was added (final concn. 0.5 mM) and the increase in fluorescence recorded. At $t = 1$ min, $CaCl_2$ was added (final concn. 1 mM). Fluorescence measurements were recorded at regular intervals.

Sedimentation-velocity experiments were carried out in a Beckman model E analytical ultracentrifuge at ~ 20 °C, the absorption optical system being used for protein concentrations < 1 mg/ml and the schlieren optical system for higher concentrations. The following protein concentrations were used: 0.6, 1.0, 1.5 and 2.0 mg/ml. The centrifuge speed was 60000 rev./min for the schlieren runs and 52000 rev./min for the u.v. ones. In the schlieren experiments both a wedge-window cell and a plain-window cell were run simultaneously, giving two schlieren patterns in the same photographic frame. The usual corrections to the apparent sedimentation coefficients were made for solvent viscosity and density, the latter quantities being taken from Zimmerman's (1960) tables. The partial specific volume of gizzard myosin was taken as 0.728 ml/g. $s^0_{20,w}$ values were calculated by extrapolation of linear plots of $s_{20,w}$ versus protein concentration to zero protein concentration.

RESULTS

Purification of gizzard myosin

Myosin was initially purified from chicken gizzard by the procedure of Persechini & Hartshorne (1983). Typical yields in the five preparations, starting from 100 g of gizzard, were 453, 365, 518, 513 and 453 mg. The data in Fig. 1(*a*) indicate that such myosin preparations contain trace amounts of contaminating calmodulin and myosin light-chain kinase, in agreement with the observations by Sobieszek (1985). These contaminants cannot be seen on overloaded gels, but can be detected by measurements of myosin phosphorylation, as shown in Fig. 1(*a*). It is desirable in a number of experimental situations to have available a preparation of myosin which is completely free of calmodulin and myosin light-chain kinase. We achieved the removal of these contaminants by subjecting this myosin to Ca^{2+}-dependent hydrophobic-interaction chromatography to remove the endogenous calmodulin, followed by calmodulin–Sepharose affinity chromatography to remove the endogenous myosin light-chain kinase. Myosin was recovered in the flow-through fractions from these columns. Yields from five preparations were 69, 71, 68, 69 and 72%. The remainder is accounted for by myosin recovered in the flow-through fractions but not included in the pooled material, and a small amount of myosin and contaminants bound to the columns in the presence of Ca^{2+} and eluted with EGTA. The pooled column-purified myosin was completely free of calmodulin and myosin light-chain kinase, and could be stoichiometrically phosphorylated by exogenous calmodulin plus myosin light-chain kinase (Fig. 1*b*).

ATPase activities of column-purified and non-purified myosins

Column-purified myosin exhibited normal actin-activated Mg^{2+}-ATPase activity, which required Ca^{2+}-dependent phosphorylation, when assayed immediately after column purification, i.e. the column-purified myosin behaved exactly like the non-purified myosin (see Fig. 2, day 0). However, the non-purified myosin retained Ca^{2+}-dependence of the actin-activated Mg^{2+}-ATPase, whereas the column-purified myosin lost this Ca^{2+}-dependence with time (Fig. 2). Both non-purified and column-purified myosins exhibited a steady loss of activity in the presence of Ca^{2+} over the 8-day period studied, but only the column-purified preparation lost Ca^{2+}-dependence. This slow transition of column-purified myosin to a Ca^{2+}-independent form is highly reproducible, having been seen in eight out of eight preparations.

Five-day-old myosins, both non-purified and column-purified, were specifically phosphorylated on the 20 kDa light chains in a Ca^{2+}-dependent manner under the ATPase assay conditions, as shown by SDS/polyacrylamide-gel electrophoresis and autoradiography (results not shown). Measurements of the stoichiometry of

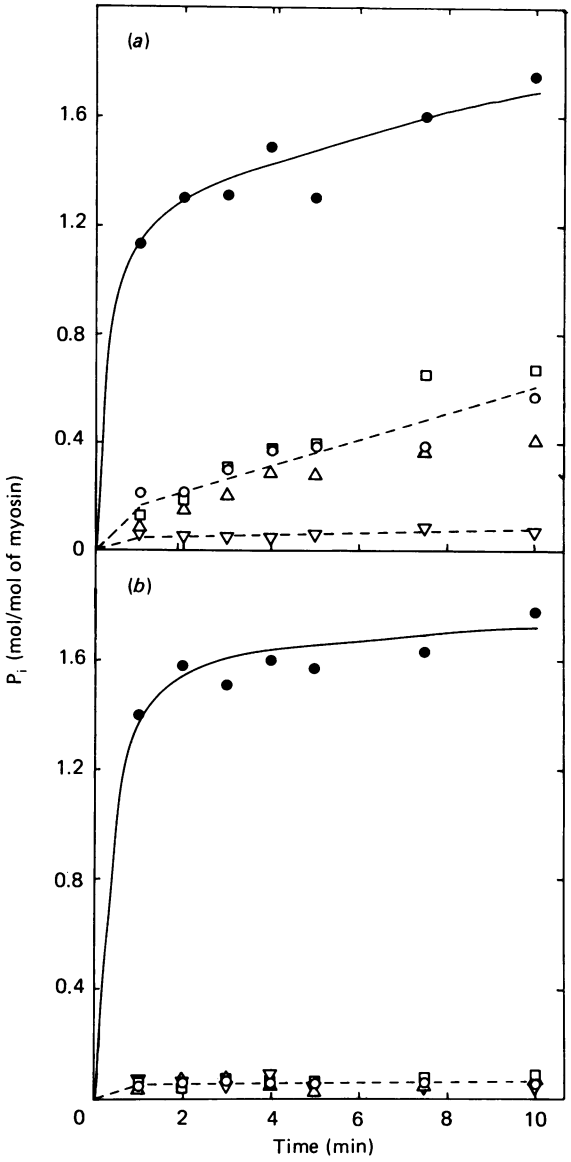

Fig. 1. Myosin preparations contain contaminating calmodulin and myosin light-chain kinase which can be removed by Ca^{2+}-dependent hydrophobic-interaction chromatography and calmodulin–Sepharose affinity chromatography

Myosin (0.5 mg/ml) was incubated at 30 °C in 25 mM-Tris/HCl (pH 7.5), 60 mM-KCl, 4 mM-$MgCl_2$, 1 mM-[γ-^{32}P]ATP (3500 c.p.m./nmol) and the following combinations: ●, 0.1 mM-$CaCl_2$ + 15 μg of calmodulin/ml + 10 μg of myosin light-chain kinase/ml; □, 0.1 mM-$CaCl_2$ + 10 μg of myosin light-chain kinase/ml; ○, 0.1 mM-$CaCl_2$ + 15 μg of calmodulin/ml; △, 0.1 mM-$CaCl_2$; ▽, 1 mM-EGTA + 15 μg of calmodulin/ml + 10 μg of myosin light-chain kinase/ml. Reaction volume was 4.0 ml. Samples (0.5 ml) were withdrawn at the indicated times for quantification of protein-bound [^{32}P]phosphate (Walsh *et al.*, 1983). (*a*) Non-purified myosin; (*b*) column-purified myosin.

phosphorylation yielded the following results: non-purified myosin, 1.74 mol of P_i/mol of myosin (+Ca^{2+}) and 0.04 mol/mol (+EGTA); column-purified myosin, 1.55 mol/mol (+Ca^{2+}) and 0.04 mol/mol (+EGTA).

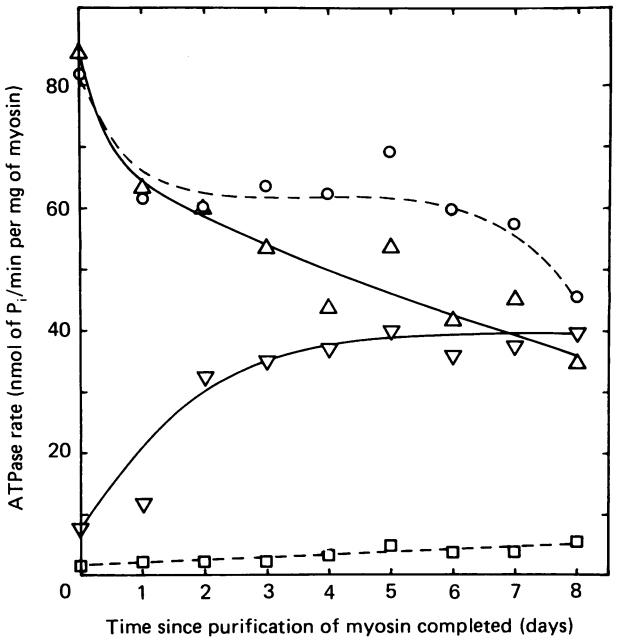

Fig. 2. Time-dependent loss of Ca^{2+}-dependence of the actin-activated Mg^{2+}-ATPase of column-purified gizzard myosin

Myosin (0.44 mg/ml), actin (0.25 mg/ml), tropomyosin (50 μg/ml), calmodulin (15 μg/ml) and myosin light-chain kinase (10 μg/ml) were incubated at 30 °C in 25 mM-Tris/HCl (pH 7.5), 80 mM-KCl, 10 mM-$MgCl_2$ and either 0.1 mM-$CaCl_2$ or 1 mM-EGTA. Reactions were initiated by the addition of [γ-^{32}P]ATP (~ 3000 c.p.m./nmol; final concn. 1 mM) in a total volume of 4.0 ml. Samples (0.5 ml) of reaction mixtures were withdrawn at 1, 2, 3, 4, 5, 7.5 and 10 min for quantification of ATPase activity as described by Ikebe & Hartshorne (1985). From the linear ATPase time courses, the ATPase rates were calculated and are plotted as a function of time since completion of the preparation of myosin. Symbols: ○, non-purified myosin + Ca^{2+}; □, non-purified myosin + EGTA; △, column-purified myosin + Ca^{2+}; ▽, column-purified myosin + EGTA.

The possibility that loss of Ca^{2+}-dependence of the actin-activated myosin Mg^{2+}-ATPase was due to a time-dependent transition from the inactive 10 S (folded) conformation to the active 6 S (extended) conformation was investigated by using five experimental approaches which are known to distinguish between these two conformers of gizzard myosin: measurements of myosin Ca^{2+}-ATPase activity in the absence of actin, measurements of myosin Mg^{2+}-ATPase activity in the absence of actin, susceptibility to papain digestion, enhancement of intrinsic tryptophan fluorescence by ATP binding, and sedimentation-velocity measurements.

Studies of myosin conformational changes

Ikebe *et al.* (1983*a*) have shown that the Ca^{2+}-ATPase activity of unphosphorylated gizzard myosin is low at low ionic strength, where myosin is in the 10 S conformation, and high at high ionic strength, where myosin is in the 6 S conformation. This is confirmed for non-purified myosin in Fig. 3. Column-purified myosin behaves like non-purified myosin immediately after

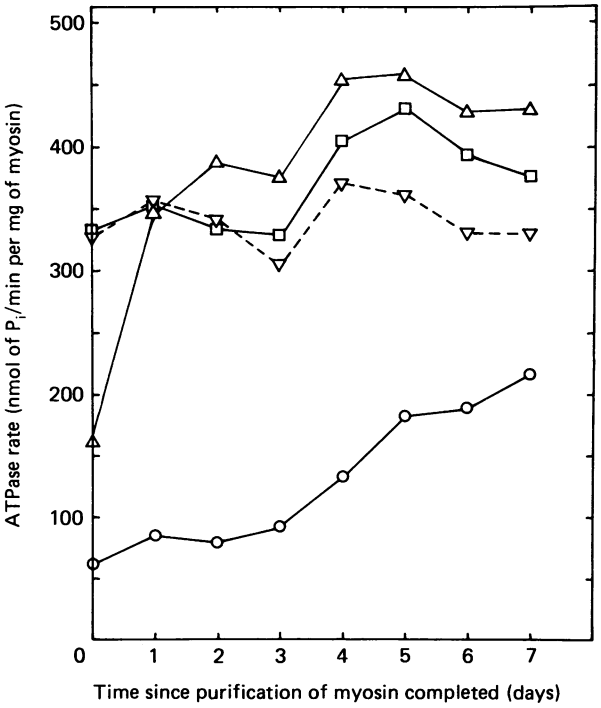

Fig. 3. Time-dependence of the Ca^{2+}-ATPase activities of non-purified and column-purified myosins at low and high ionic strength

Myosin (0.15 mg/ml) was incubated at 30 °C in 30 mM-Tris/HCl (pH 7.5), 10 mM-$CaCl_2$ and either 0.1 M- or 0.35 M-KCl. Reactions were initiated by the addition of [γ-^{32}P]ATP (~ 4000 c.p.m./nmol; final concn. 1 mM) in a reaction volume of 3.0 ml. Samples (0.4 ml) of reaction mixtures were withdrawn at 1, 2, 3, 4, 5, 7.5 and 10 min for quantification of ATPase activity. From the linear ATPase time courses, the ATPase rates were calculated and are plotted as in Fig. 2. Symbols: ○, non-purified myosin + 0.1 M-KCl; □, non-purified myosin + 0.35 M-KCl; △, column-purified myosin + 0.1 M-KCl; ▽, column-purified myosin + 0.35 M-KCl.

column purification, but rapidly changes to a form which exhibits high Ca^{2+}-ATPase activity at both low and high ionic strength, suggesting that column-purified myosin exists in the 10 S conformation immediately after column purification but subsequently changes spontaneously to the 6 S conformation. Similar results were obtained from measurements of the myosin Mg^{2+}-ATPase at low and high ionic strength as a function of time since column purification (results not shown).

It has also been established that the 6 S and 10 S conformers of gizzard myosin display differential susceptibility to digestion by papain (Onishi & Watanabe, 1984; Ikebe & Hartshorne, 1984). In agreement with these findings, we observed that phosphorylated non-purified myosin (6 S conformation) was more susceptible to digestion by papain than was non-phosphorylated, non-purified myosin (10 S conformation). This was true whether myosin was digested 1 day or 6 days after preparation (Figs. 4*a*, 4*c*, 4*e* and 4*g*). On the other hand, even 1 day after purification the non-phosphorylated column-purified myosin displayed a papain-digestion pattern intermediate between the 10 S and 6 S conformations (Fig. 4*b*). At 6 days after purification the digestion pattern of the non-phosphorylated column-purified myosin (Fig. 4*f*) was very similar to that of the 6 S conformer. As predicted, the phosphorylated column-purified myosin exhibited a digestion pattern typical of the 6 S conformer on both day 1 and day 6 (Figs. 4*d* and 4*h*). Whereas the patterns of digestion of the heavy chain of non-phosphorylated and phosphorylated column-purified myosin after transition are indistiguishable, there is a difference in digestion of the 20 kDa light chain (cf. Figs. 4*f* and 4*h*). In the non-phosphorylated state the 20 kDa light chain is degraded to a 16.4 kDa fragment, whereas in the phosphorylated form a 17.8 kDa peptide is generated. The conformation or location of the 20 kDa light chain is therefore affected by phosphorylation of column-purified myosin after transition, but this change is not required for activation of the myosin.

Ikebe *et al.* (1983*b*) have shown that ATP binding to gizzard myosin enhances the intrinsic tryptophan fluorescence and that transition from the 10 S (folded) to the 6 S (extended) conformation is accompanied by a decrease in fluorescence. We confirmed the ATP-induced enhancement of fluorescence of non-purified myosin to a higher value with 10 S myosin than with 6 S myosin. Phosphorylation of 10 S myosin to convert it into the 6 S conformer was accompanied by a fall in fluorescence to that observed with 6 S myosin (results not shown). On the other hand, column-purified myosin 7 days after purification, whether at low or high ionic strength, phosphorylated or non-phosphorylated, gave fluorescence data characteristic of the 6 S monomer.

Sedimentation-velocity measurements of phosphorylated and non-phosphorylated column-purified myosin 6 days after purification each gave sedimentation coefficients ($s^0_{20,w}$) of 6.3 S. As expected, the phosphorylated non-purified myosin exhibited a sedimentation coefficient of 6.0 S, and non-phosphorylated non-purified myosin a sedimentation coefficient of 9.75 S.

All these data support the conclusion that column-purified myosin undergoes a time-dependent conformational transition from the 10 S (folded) to the 6 S (extended form). Upon completion of this conformational transition, the myosin no longer requires phosphorylation for actin activation of its Mg^{2+}-ATPase activity. One possible explanation for this transition was slow oxidation of the myosin after column purification, in spite of the presence of 0.2 mM-dithiothreitol, particularly in light of the fact that Chandra *et al.* (1985) demonstrated loss of Ca^{2+}-dependence of the actin-activated Mg^{2+}-ATPase after modification of thiol groups in smooth-muscle myosin by *N*-ethylmaleimide. This proved to be the case, since the transition could be prevented by storing the myosin under N_2 or could be reversed by the addition of 5 mM-dithiothreitol (Table 1). It was still unclear why column purification should induce this conformational transition, and we found that the transition still occurred if myosin was subjected only to Ca^{2+}-dependent hydrophobic-interaction chromatography or calmodulin–Sepharose affinity chromatography (Table 1). The only difference between the storage conditions for non-purified and column-purified myosins was that the latter included 0.1 mM-$CaCl_2$. We found that addition of 1 mM-EGTA to column-purified myosin immediately after column purification did not prevent the transition (Table 1). Storage of non-purified myosin in the presence of 0.1 mM-$CaCl_2$ led to a partial

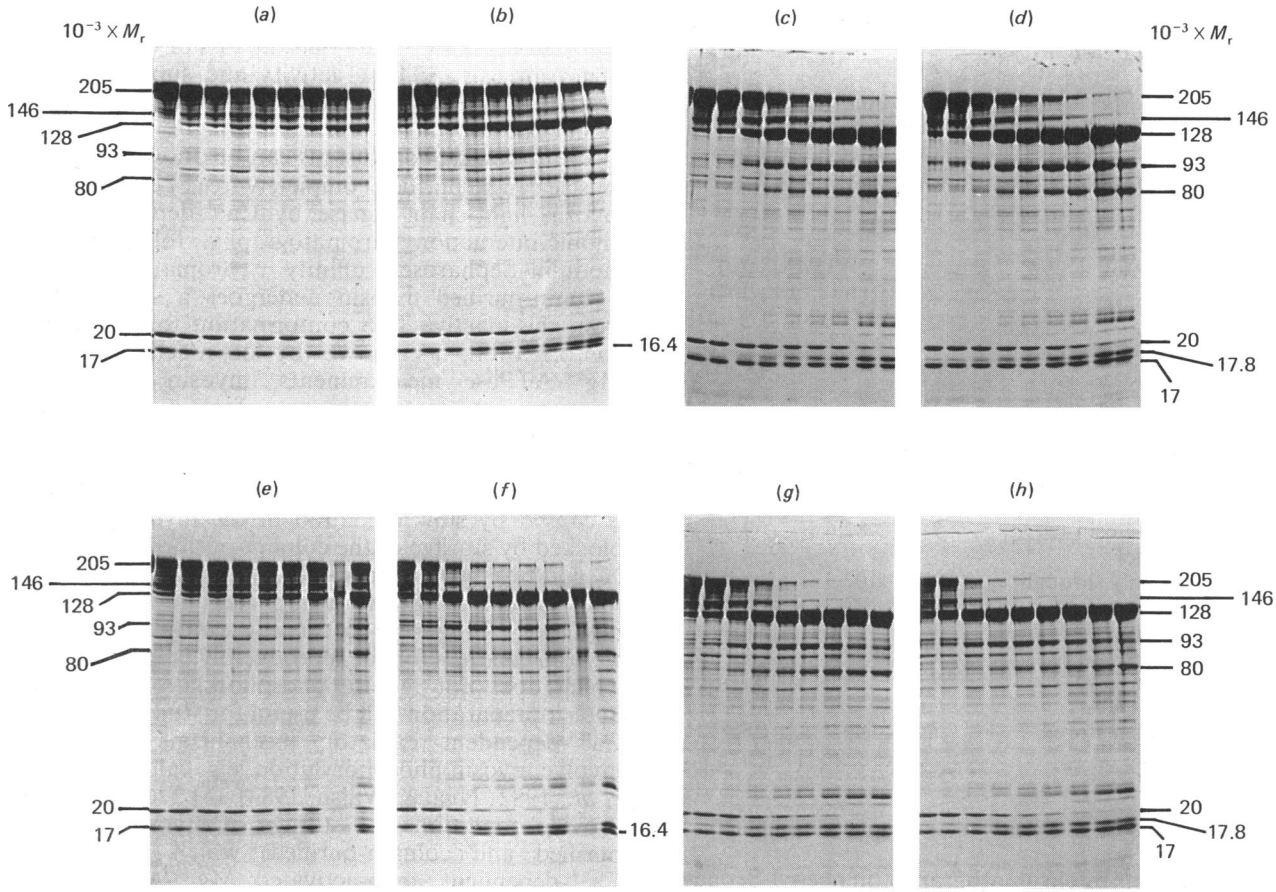

Fig. 4. Papain digestion of phosphorylated and non-phosphorylated forms of non-purified and column-purified myosins

Non-purified and column-purified myosins (1 day and 6 days old) were phosphorylated as follows: myosin (1.5 mg/ml) was incubated for 10 min at 35 °C with 15 μg of calmodulin/ml and 15 μg of myosin light-chain kinase/ml in 30 mM-Tris/HCl (pH 7.5), 1 mM-$MgCl_2$, 0.16 mM-$CaCl_2$ and 1 mM-[γ-^{32}P]ATP ($\sim$ 7000 c.p.m./nmol). Phosphorylated myosins and non-phosphorylated myosins were then immediately digested with activated papain as described in the Materials and methods section. Phosphorylation was determined to be 1.73 mol of P_i/mol of non-purified myosin and 1.79 mol of P_i/mol of column-purified myosin. Samples (50 μl) of digestion mixtures were withdrawn at 1, 2, 5, 10, 15, 20, 30 45 and 60 min, added to an equal volume of 50 mM-Tris/HCl (pH 6.8)/1% (w/v) SDS/30% (v/v) glycerol/0.01% (w/v) Bromophenol Blue/2% (v/v) 2-mercaptoethanol and immersed in a boiling-water bath for 2 min. Samples (20 μl) containing 10 μg of protein were subjected to 0.1% SDS/7.5–20%-polyacrylamide-gradient-slab-gel electrophoresis as described in the Materials and methods section. Each panel represents a time course of digestion: (*a*) non-phosphorylated non-purified myosin (day 1); (*b*) non-phosphorylated column-purified myosin (day 1); (*c*) phosphorylated non-purified myosin (day 1); (*d*) phosphorylated column-purified myosin (day 1); (*e*) non-phosphorylated non-purified myosin (day 6); (*f*) non-phosphorylated column-purified myosin (day 6); (*g*) phosphorylated non-purified myosin (day 6); (*h*) phosphorylated, column-purified myosin (day 6). Numbers indicate M_r values ($\times 10^{-3}$) based on the mobilities of M_r marker proteins electrophoresed simultaneously.

(55%), but not total, loss of Ca^{2+}-dependence of the actin-activated Mg^{2+}-ATPase in 5 days (Table 1).

DISCUSSION

It is now widely accepted that the primary Ca^{2+}-dependent mechanism for the regulation of smooth-muscle contraction involves the reversible phosphorylation of myosin (Adelstein & Eisenberg, 1980; Walsh & Hartshorne, 1982; Walsh, 1985). Biochemical evidence has indicated that this phosphorylation at serine-19 of the 20 kDa light chain induces an important conformational change in the head-neck region of the myosin molecule, which enhances the actin-activated myosin Mg^{2+}-ATPase activity (Ikebe & Hartshorne, 1984, 1985). In order to study the detailed mechanisms of regulation by phosphorylation, it is very useful to have available a preparation of smooth-muscle myosin which is completely free of calmodulin and myosin light-chain kinase. Myosin preparations made by standard procedures (e.g. Persechini & Hartshorne, 1983) are contaminated with trace amounts of both calmodulin and myosin light-chain kinase, although Chacko *et al.* (1977) have reported the use of gel filtration to separate myosin light-chain kinase and phosphatase activities from smooth-muscle myosin. We reasoned that it should be relatively easy to remove calmodulin and myosin light-chain kinase from myosin preparations by exploiting the Ca^{2+}-dependent interaction of calmodulin with a hydrophobic matrix and the Ca^{2+}-dependent affinity of myosin light-chain kinase for immobilized calmodulin, and were successful in removing

Table 1. Actin-activated Mg^{2+}-ATPase activities of various forms of gizzard myosin

Actin-activated Mg^{2+}-ATPase rates of non-purified and column-purified myosins, treated under a variety of conditions, were measured immediately after preparation (day 0) or 5 days later under conditions described in the legend to Fig. 2

Myosin	Day	ATPase rate (nmol of P_i/min per mg of myosin) $+Ca^{2+}$	$-Ca^{2+}$
Non-purified myosin stored under N_2	5	95.3	1.9
Column-purified myosin stored under N_2	5	79.0	6.4
Column-purified myosin + 5 mM-dithiothreitol	5	82.6	9.7
Myosin purified only through calmodulin–Sepharose	0	102.3	6.7
	5	37.2	34.0
Myosin purified only through phenyl-Sepharose	0	112.0	6.5
	5	23.9	21.4
Column-purified myosin stored in 1 mM-EGTA	5	39.0	39.5
Non-purified myosin stored in 0.1 mM-$CaCl_2$	5	49.9	27.0

both contaminants by chromatography on phenyl-Sepharose followed by calmodulin–Sepharose in the presence of Ca^{2+}. The properties of this myosin immediately after purification were indistinguishable from those of non-purified myosin, except for the absence of calmodulin and myosin light-chain kinase. However, the column-purified myosin underwent a time-dependent transition from a form which required phosphorylation for actin activation of its Mg^{2+}-ATPase to a form which did not. We have demonstrated that this transition involves a slow conversion of the column-purified myosin from the 10 S (inactive) into the 6 S (active) conformation. This conformational transition was caused by oxidation of the column-purified myosin, since it could be prevented by storing the myosin under N_2 immediately after column purification, or reversed by the addition of 5 mM-dithiothreitol. The integrity of thiol groups is clearly important in maintaining the regulatory properties of gizzard myosin. Other reports (Seidel, 1979; Onishi, 1985; Chandra *et al.*, 1985) have indicated that blocking of thiol groups in gizzard myosin can lead to a loss of Ca^{2+}- and phosphorylation-dependence of the actin-activated Mg^{2+}-ATPase activity.

Sparrow *et al.* (1970) reported a complete loss of Ca^{2+}-sensitivity of ATPase activity and superprecipitation of actomyosin prepared from hog carotid arteries when the actomyosin was stored for 3 weeks or more at 0 °C in the absence of reducing agent. This loss of Ca^{2+}-sensitivity was prevented by dithiothreitol. They suggested that the observed loss of Ca^{2+}-sensitivity in the absence of dithiothreitol may be due to inactivation of the regulatory proteins, troponin and tropomyosin, by oxidation of thiol groups. However, in the light of current understanding of the mechanism of regulation of smooth-muscle contraction, in particular the absence of troponin from smooth muscle, and our observations reported here, it appears that the loss of Ca^{2+}-sensitivity of actomyosin ATPase activity and superprecipitation described by Sparrow *et al.* (1970) was due to myosin oxidation.

The following conclusions can be drawn: (1) smooth-muscle can be purified completely free of calmodulin and myosin light-chain kinase by Ca^{2+}-dependent hydrophobic-interaction chromatography, followed by calmodulin–Sepharose affinity chromatography. (2) Column-purified myosin undergoes a slow transition from the inactive 10 S conformation to the active 6 S conformation, as shown by actin-activated myosin Mg^{2+}-ATPase measurements, myosin Mg^{2+}-ATPase measurements in the absence of actin, myosin Ca^{2+}-ATPase measurements, papain digestion, enhancement of intrinsic tryptophan fluorescence by ATP binding, and sedimentation-velocity measurements. (3) This transition is caused by slow oxidation of the myosin, since it is blocked by storage of the column-purified myosin under N_2 and reversed by 5 mM-dithiothreitol. The integrity of thiol groups is therefore important in preserving the regulatory properties of smooth muscle myosin. (4) Myosin can therefore be reproducibly prepared in a form which does not require phosphorylation for activity. Such a preparation will be useful for studying secondary Ca^{2+}-dependent regulatory mechanisms which do not involve myosin phosphorylation, e.g. caldesmon (Sobue *et al.*, 1982; Ngai & Walsh, 1984). (5) These results also provide conditions for storage of myosin (both non-purified and column-purified) which retain a high Ca^{2+}-dependent actin-activated Mg^{2+}-ATPase for at least 2 weeks, i.e. 10 mM-Tris/HCl (pH 7.5)/0.3 M-KCl/0.2 mM-dithiothreitol, under N_2. (6) These observations indicate the possibility that the regulatory properties of isolated smooth-muscle myosin may undergo unexpected changes which could lead to erroneous conclusions about the mechanisms of regulation of smooth-muscle contraction. (7) These results lend further support to the notion that conformation dictates the activity of smooth-muscle myosin.

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