

Phosphorylation and actin activation of brain myosin

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A method is described for obtaining brain myosin that shows significant actin activation after phosphorylation with chicken gizzard myosin light chain kinase. Myosin with this activity could be obtained only via the initial purification of brain actomyosin. The latter complex, isolated by a method similar to that used for smooth muscle, contained actin, myosin, tropomyosin of the non-muscle type and another actin-binding protein of ~100 000 daltons. From the presence of a specific myosin light chain kinase and phosphatase in brain tissue it is suggested that the regulation of actin-myosin interaction operates via phosphorylation and dephosphorylation of myosin.

Key words: brain actomyosin/myosin phosphorylation/Ca-dependent regulation

Introduction

The presence of actomyosin-like proteins in diverse non-muscle systems has led to the general presumption that the interaction between actin and myosin plays a role in various forms of cellular motility such as platelet contraction, cytoplasmic streaming, axoplasmic transport, phagocytosis and cell locomotion (for reviews, see Pollard and Weihing, 1974; Hitchcock, 1977; Korn, 1978; Trifaro, 1978; Hatano *et al.*, 1979). It seems likely that the regulation of non-muscle actomyosin systems involves a number of different mechanisms. However, these control mechanisms have yet to be properly characterised. Current concepts of regulation in non-muscle cells stem from the systems discovered in muscle and can be separated into either regulation *via* actin, or 'actin-linked regulation' as in skeletal muscle (Shibata *et al.*, 1972; Cohen *et al.*, 1973; Puszkin *et al.*, 1974; Mahendran and Berl, 1976) or myosin-linked regulation operating *via* phosphorylation and dephosphorylation of myosin, as in smooth muscle (Adelstein and Conti, 1975; Scordilis and Adelstein, 1978; Yerna *et al.*, 1978; Trotter and Adelstein, 1979; Scholey *et al.*, 1980). In this context the regulatory mechanism of brain actomyosin is very controversial. On the one hand, the isolation of a brain troponin complex was reported (Mahendran and Berl, 1976, 1978, 1979) while on the other the presence of a specific myosin light chain kinase could be demonstrated (Dabrowska and Hartshorne, 1978; Hathaway *et al.*, 1981).

In the present study we describe a new and very reliable procedure for the purification of brain actomyosin and its components. This procedure also enabled us to purify myosin that was highly actin-activatable in the presence of myosin light chain kinase. It is concluded that in brain tissue, as in

smooth muscle, myosin phosphorylation represents an obligatory step in actin-myosin interaction.

Results

Isolation and composition of actomyosin

The procedure for the isolation and purification of pig brain actomyosin was developed from methods found most suitable for the preparation of smooth muscle contractile proteins (Small and Sobieszek, 1982) and comprised the following main steps. (i) Removal of most of the soluble proteins prior to actomyosin extraction by extensive washing of the tissue first in the presence and then in the absence of Triton X-100 (Figure 1 A,B). (ii) Extraction of actomyosin at low ionic strength in the presence of ATP and its precipitation between 35 and 55% ammonium sulphate saturation (Figure 1 C,F). (iii) Purification of actomyosin by its solubilization at high ionic strength in the presence of ATP and its subsequent precipitation by 50 mM MgCl₂ (Figure 1 G). (iv) Solubilization of remaining impurities at 25% ammonium sulphate saturation under which conditions the precipitated actomyosin remains insoluble (Figure 1 H).

By this procedure we routinely obtained 10–15 mg of actomyosin per 100 g of fresh brain tissue. On SDS-polyacrylamide gels the major components characteristic for actomyosin can be recognized after step (iii). A gel of purified actomyosin (step iv, Figure 1 H) shows bands corresponding to myosin, actin, tropomyosin and an additional protein of ~100 000 dalton. The latter component was identified as an actin-binding protein since it co-migrated with actin on a gel filtration column (Figure 1 J) and could be sedimented with brain actin by high speed centrifugation (Figure 1 K). The origin of the actomyosin from brain tissue and not from possible contaminants of smooth muscle was most clearly indicated by the presence of tropomyosin of only the non-muscle type. As shown in Figure 1 G and H its polypeptide chain weight was ~30 000 daltons, as previously reported (Cohen and Cohen, 1972; Fine *et al.*, 1973; Blitz and Fine, 1974; Fine and Blitz, 1975; Masaki, 1975). The apparently low tropomyosin content of purified actomyosin may be attributed to the last purification step; namely, precipitation at 25% ammonium sulphate saturation. Under these conditions most of the tropomyosin is removed from smooth muscle actomyosin (Sobieszek and Small, 1977). Indeed, the 25% ammonium sulphate supernatant of brain actomyosin contained the 30 000-dalton band which could be identified as tropomyosin by two-dimensional gel electrophoresis (Carmon *et al.*, 1978; Barylko *et al.*, 1982) and by its binding to reconstituted skeletal muscle actomyosin (data not shown).

Myosin and its enzymatic properties

As indicated in Materials and methods, myosin could be purified either directly from the crude extract (Figure 1 C,D) or from actomyosin (Figure 1 H) by its separation on gel filtration in the presence of MgATP (Figure 2). In both cases pure myosin was eluted in the first peak (Figure 1 E and I) and after precipitation, ~5 mg of myosin per 100 g of tissue, was obtained. The myosin was composed of a heavy chain of

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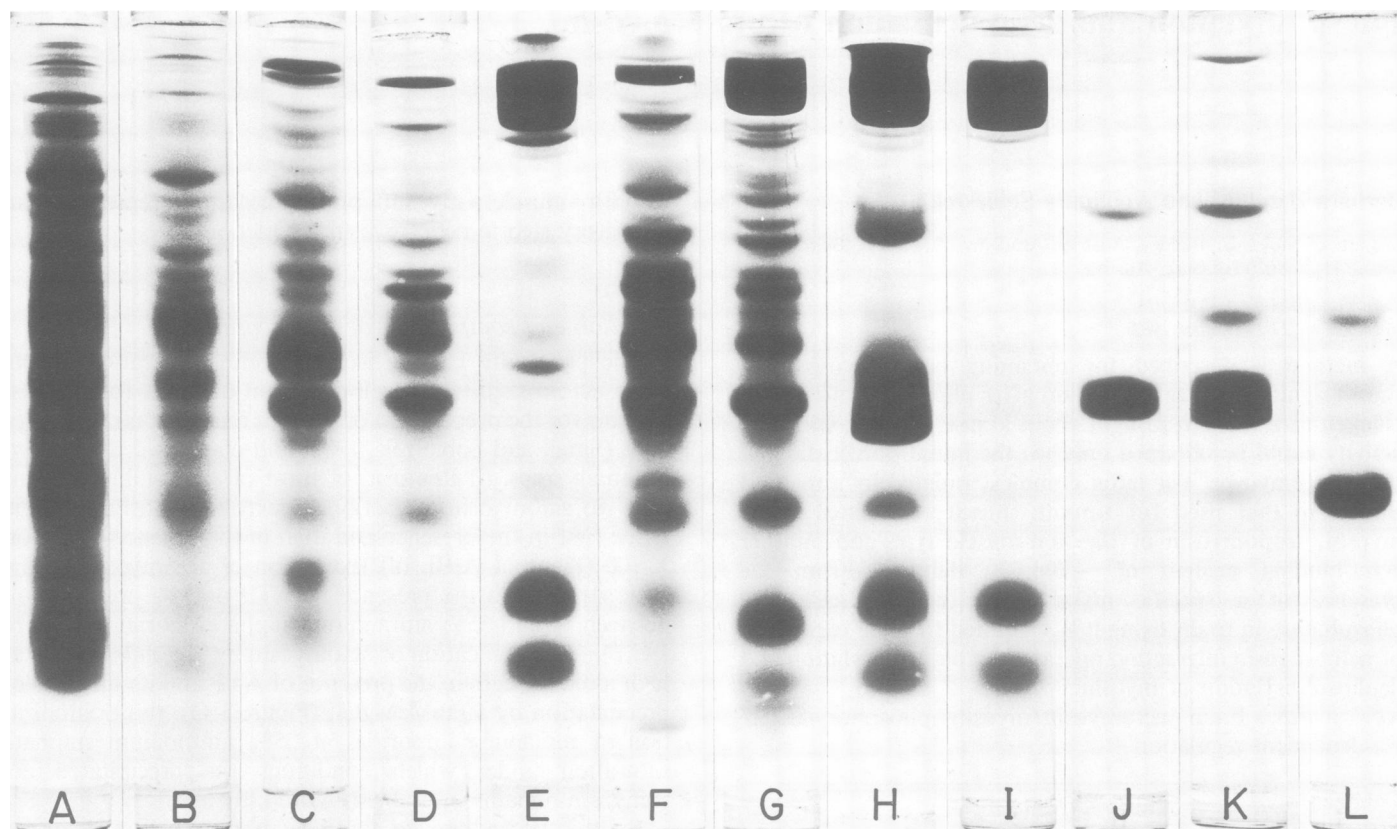


Fig 1. SDS-gels of the major steps in the purification of contractile proteins from pig brain. For more details see Materials and methods. (A) and (B) First and third wash; (C) LAMES extract; (D) 45–55% ammonium sulphate fraction of (C); (E) purified myosin obtained after gel filtration of (D); (F) 35–55% ammonium sulphate fraction of (C); (G) crude actomyosin obtained by $MgCl_2$ precipitation of (F); (H) purified actomyosin; (I) myosin purified by gel filtration of actomyosin (H) in the presence of (Mg)ATP (Sepharose 4B 1st peak); (J) actin component of actomyosin (2nd peak of the Sepharose 4B, concentrated); (K) F-actin; pellet obtained by high speed centrifugation of (J); (L) crude tropomyosin obtained by one ammonium sulphate (40–55%) fractionation and one isoelectric precipitation (pH 4.6) of 1st wash (A).

200 000 daltons, typical for muscle myosin, and two species of light chains of 19 000 and 17 000 daltons. After gel filtration, gizzard and brain myosins eluted at exactly the same position indicating that the two native molecules are very similar in size.

Under conditions of high ionic strength, brain myosin exhibited an ATPase activity characteristic of muscle myosins. The potassium-activated activity was $0.14 \pm 0.01 \mu\text{mol } P_i/\text{mg}/\text{min}$ and was inhibited 3-fold by $CaCl_2$ while the inhibition by $MgCl_2$ was ~30-fold.

Phosphorylation of myosin

Incubation of purified actomyosin and myosin with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ did not yield any significant level of phosphorylation. This could be correlated with the nature of the purification method which would be expected to promote the removal of myosin light chain kinase. Myosin phosphorylation was readily effected by the addition of exogenous myosin light chain kinase purified from chicken gizzard. Analysis of ^{32}P -phosphorylated actomyosin and myosin by SDS-polyacrylamide gel electrophoresis showed that the only component labelled was the 19 000-dalton light chain (Figure 3). Quantitative determination revealed that in both preparations only 1 mol of ^{32}P was incorporated per mol of myosin.

Myosin light chain kinase and phosphatase activity

In view of the above results it was necessary to confirm that crude preparations of actomyosin and myosin contained a specific myosin light chain kinase and phosphatase. It was

found (Table I) that the initial fraction used for preparation of actomyosin or myosin (35–55 or 45–55% saturation of ammonium sulphate fraction) was rich in myosin light chain kinase activity, as shown by the phosphorylation of smooth muscle myosin P-light chain. After precipitation of actomyosin from this fraction by 50 mM $MgCl_2$, the main kinase activity remained in the supernatant and only negligible activity was co-precipitated with actomyosin. Purified myosin was completely devoid of kinase activity. When crude myosin was subjected to gel filtration the kinase activity was found in the second peak (see Figure 2). The endogenous kinase activity was higher in the presence of calcium ions, as indicated in Table I, and showed a Ca-sensitivity of the order of 65%. The distribution of myosin light chain phosphatase activity was similar to that for the kinase (Table I). As for the myosin light chain kinase, the phosphatase activity was found mainly in the supernatant after precipitation of actomyosin by 50 mM $MgCl_2$ and in the second peak obtained by gel filtration. The phosphatase was Ca-insensitive with regard to the dephosphorylation of ^{32}P -labelled gizzard myosin light chain.

Actin-activated ATPase activity

In a 'regulated' actomyosin system the actin-activated ATPase activity requires the presence of micromolar concentrations of calcium ions. The ATPase activities of crude and purified brain actomyosin measured in the presence and absence of Ca^{2+} are shown in Figure 4 A (first points of the curves on the ordinate). In both cases the activities are ex-

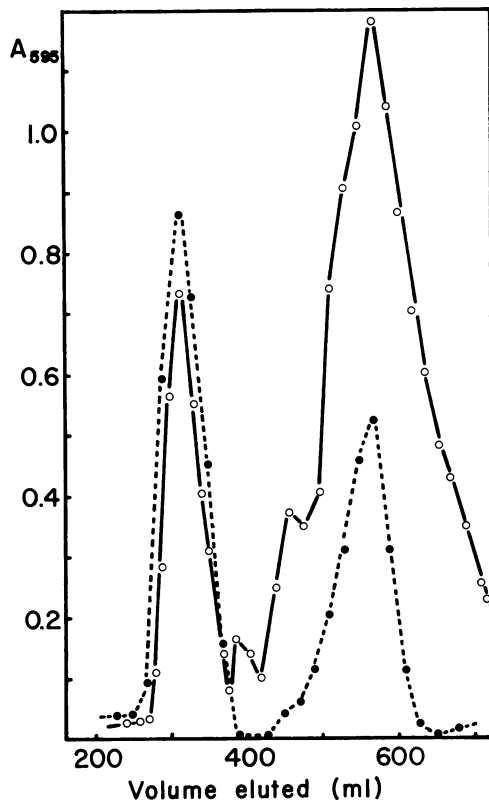


Fig. 2. Gel filtration of crude myosin (solid line) and actomyosin (broken line) in the presence of MgATP. A Sepharose 4B-CL column (3.2 cm x 95 cm) was equilibrated with myosin column solution (see text) and eluted at ~40 ml/h. 250 μ l of each fraction was taken for protein determination using the method of Bradford in BioRad assays.

tremely low, 2–4 nmol/mg/min, and may represent only the residual activity of brain myosin. The addition of purified smooth muscle myosin light chain kinase resulted in a 3- to 4-fold stimulation of this activity but only under conditions where the kinase is active, that is in the presence of Ca^{2+} . That this corresponded to actin-activated ATPase activity is shown in Figure 4 B. Thus, the addition of purified rabbit skeletal muscle F-actin resulted in a further 2- to 3-fold increase in activity. Significantly, the crude actomyosin, having a lower actin content (Figure 1 G) was stimulated to a greater extent than purified actomyosin which showed a higher actin to myosin ratio (Figure 1 H). Figure 5 shows a similar experiment in which purified brain myosin was recombined with rabbit actin and gizzard myosin light chain kinase. The latter was added in an amount to ensure maximal phosphorylation of myosin within the first 20–30 s (insert). This activation time is negligible in comparison with the time of the ATPase assays e.g., 10 min. As is shown, there was a 10-fold activation of myosin ATPase by actin in the presence of Ca^{2+} and the kinase. This level of activity which is comparable with that for smooth muscle actomyosin (Sobieszek and Small, 1976; Chacko *et al.*, 1977; Litten *et al.*, 1979) could be achieved only with myosin purified from actomyosin. Myosin obtained directly from the first extract showed not more than half this activity, presumably due to some degradation during purification (see Discussion).

Discussion

The present report provides the first clear evidence that myosin from brain is regulated, with respect to its interaction

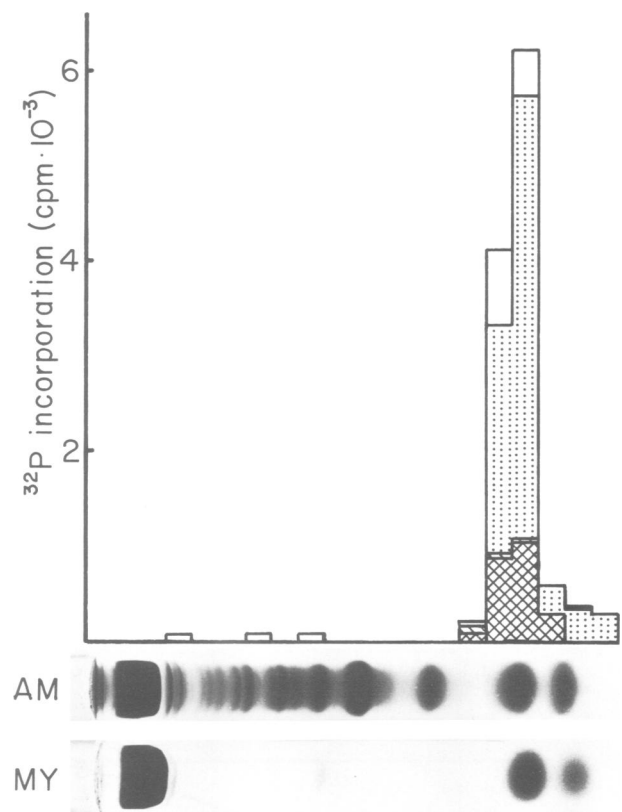


Fig. 3. Phosphorylation of brain myosin (clear area) and crude actomyosin (dotted area) in the presence of Ca^{2+} , the chicken gizzard kinase and $[\gamma\text{-}^{32}\text{P}](\text{Mg})\text{ATP}$. Note that only the 19 000-dalton light chain of myosin becomes phosphorylated. In the absence of Ca^{2+} the phosphate incorporation for both preparations (cross-hatched area) is ~10% of that with Ca^{2+} present.

Table 1. Relative myosin light chain kinase and phosphatase activities of the major fractions obtained during purification of brain actomyosin and myosin

Fraction	Myosin light chain kinase (c.p.m. ^{32}P incorporation into L_{20})		Myosin light chain phosphatase (c.p.m. ^{32}P release from L_{20})
	CaCl_2	EGTA	
35–55% fraction of LAMES extract	7100	2500	–
Crude actomyosin (CAM)	4350	2400	4400
50 mM MgCl_2 supernatant of CAM	12 300	4300	15 500
Purified myosin	670	0	0
2nd peak from column	20 900	12 300	17 100

Phosphorylation and dephosphorylation was assayed as described in Materials and methods. For determination of kinase activity 12 μ l of each fraction was used for one assay. For phosphatase activity 10 μ l of each fraction and 30 μ l of $[\text{L}_{20}\text{-}^{32}\text{P}]$ (45 000 c.p.m.) were used for one assay.

with actin, *via* a phosphorylation of one of its light chains. This demonstration depended in the main on obtaining myosin both in sufficient quantities and in a fresh and intact form. In these latter respects the purification of myosin *via* actomyosin was found to be superior to any other method. Thus, myosin purified directly from the initial extract, even after extensive washing of the tissue to remove soluble contaminants, was activated to a much lower extent by actin than

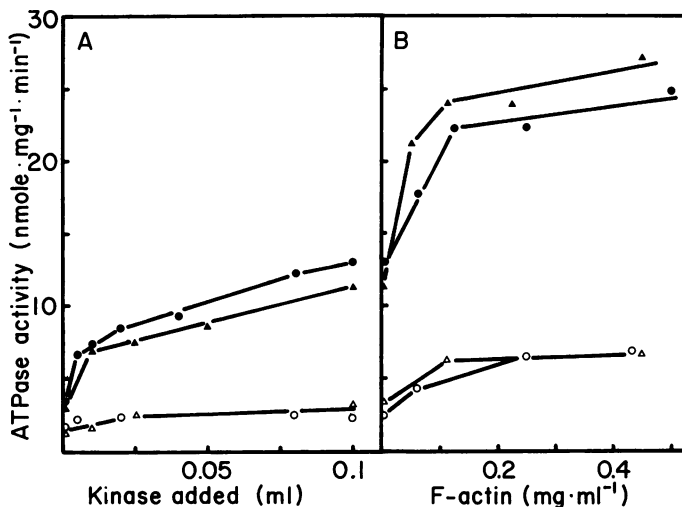


Fig. 4. Actin-activated ATPase activity of crude (triangles) and purified (circles) brain actomyosin. (A) Activation by purified chicken gizzard myosin light chain kinase. (B) Activation by added rabbit skeletal muscle F-actin. In this assay the kinase concentration was the same as in the last points of (A). Actomyosin concentration was 0.6 mg/ml. Closed and open symbols correspond to the activities in the presence and absence of Ca^{2+} , respectively.

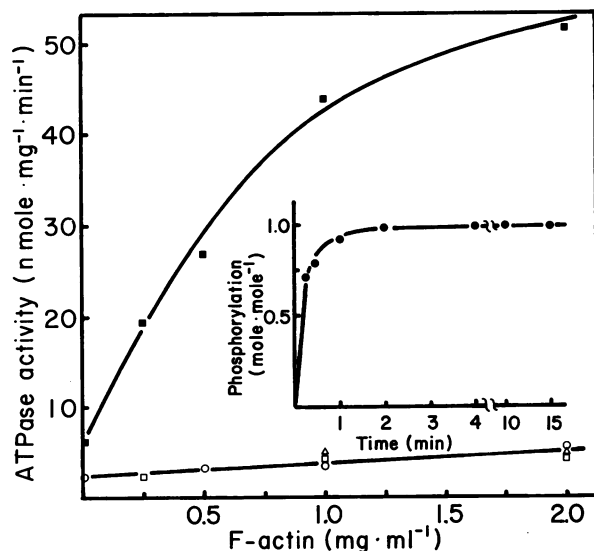


Fig. 5. Activation of brain myosin by skeletal muscle F-actin. The activation takes place only in the presence of the kinase and Ca^{2+} (solid squares). Lower curve shows the activation in the absence of kinase with or without Ca^{2+} . Assay time 10 min. Myosin concentration was 0.6 mg/ml. **Insert:** time course of myosin phosphorylation under conditions as for the ATPase measurements. Note that maximal phosphorylation (1 mol of ^{32}P incorporated per mol of myosin) is obtained after 1 min.

myosin obtained from actomyosin. We attribute this difference to a protection, presumably against proteolysis, by the binding of myosin to actin in the actomyosin complex. Brain myosin purified by the present method showed only two light chain species of 19 000 and 17 000 daltons, respectively, consistent with the recent report of Hobbs and Frederiksen (1980). Other bands of 23 000 daltons (Burrige and Bray, 1975) and 18 000 daltons (Kuczmarski and Rosenbaum, 1979) were not observed.

In contrast to earlier studies (Mahendran and Berl, 1976, 1978, 1979) we could detect no troponin-like protein in either

crude or purified brain actomyosin preparations or in the actin-rich fractions obtained by gel filtration (Figure 2, second peak). The only low mol. wt. bands observed were those of myosin light chains or tropomyosin. Concentration of the actin-containing fractions and sedimentation at high speed yielded a pellet that contained F-actin with no bands below actin except traces of tropomyosin. Similarly, in crude tropomyosin preparations, low mol. wt. bands characteristic of troponin were absent (Figure 1 L). The presence of only one type of tropomyosin of 30 000 daltons characteristic of non-muscle systems (Cohen and Cohen, 1972; Fine *et al.*, 1973; Blitz and Fine, 1974; Fine and Blitz, 1975; Masaki, 1975) excludes the possibility of significant contamination by smooth muscle actomyosin originating from the small blood vessels in brain. The function of tropomyosin in the regulation of non-muscle actin-myosin interaction is not currently understood. However, from more recent studies on the effect of muscle and non-muscle tropomyosin on the activity of smooth muscle actomyosin (Sobieszek and Small, 1981; Sobieszek, 1982) it is likely that it plays a modulatory role. As for smooth muscle actomyosin, brain tropomyosin was found to effect an increase in the actin-activated ATPase activity of brain actomyosin (data not shown). However, enzyme kinetic analyses of the brain actin-myosin interaction are needed in order to establish the exact nature of the modulation by brain tropomyosin.

The discovery of Ca-dependent myosin phosphorylation in smooth muscle (Sobieszek, 1977) stimulated a new series of studies on the regulation of actin-myosin interaction in non-muscle systems. The similarity between non-muscle and smooth muscle myosins became evident not only from their common light chain patterns but also from the finding that the phosphorylation of myosin was a general prerequisite for its interaction with actin. This could be shown for myosins from blood platelets (Adelstein and Conti, 1975), BHK 21 cells (Yerna *et al.*, 1978), proliferative myoblasts (Scordilis and Adelstein, 1978) macrophages (Trotter and Adelstein, 1979) and thymus tissue (Scholey *et al.*, 1980). The Ca^{2+} sensitivity of this interaction derives from the Ca-dependence of the kinase required for myosin light chain phosphorylation.

In the present study we could identify myosin light chain kinase activity as well as myosin light chain phosphatase activity in the brain extracts, indicating that these enzymes regulate, as in smooth muscle, the phosphorylation of myosin. Unfortunately, these two regulatory enzymes were not co-purified with actomyosin and as yet we have not developed procedures for their isolation. From the cross-reactivity of the smooth muscle and non-muscle kinases with substrates such as isolated light chain (Scordilis and Adelstein, 1978; Hathaway and Adelstein, 1979; Trotter and Adelstein, 1979) or intact myosins (unpublished data and the present study) it is clear that the non-muscle and smooth muscle enzymes are more closely related than the corresponding enzymes from skeletal muscle. Using smooth muscle myosin light chain kinase the rate of brain myosin phosphorylation is very rapid provided that sufficient amounts of the kinase are present. Attempts to slow the phosphorylation rate by decreasing the amount of the kinase lead to a negligible level of phosphorylation, possibly due to denaturation of the kinase (Pearson *et al.*, 1982). Thus, it was not possible in a simple way to test the relationship between the extent of phosphorylation of brain myosin and the actin-activated ATPase activity. As indicated in Figure 5, the extent of

phosphorylation was never more than ~1 mol/mol of myosin. This implies that only one of the myosin heads becomes phosphorylated under the assay conditions. Possibly the other head remains phosphorylated during myosin purification and this myosin is only minimally activated by actin, as for smooth muscle myosin (Persechini and Hartshorne, 1981).

The activation of myosin *via* a phosphorylation is an indirect regulation mechanism operating through a specific kinase. Since it is now clear that the troponin C-like protein isolated from brain is calmodulin (Drabikowski *et al.*, 1977), a troponin-dependent modulation at the level of brain thin filaments can be excluded. The interesting effects of phosphorylation of the myosin light chain kinase on its activity (Adelstein *et al.*, 1978; Conti and Adelstein, 1980) imply that some modulation of the regulation process may be expressed at the level of the kinase. Further studies are needed to clarify these effects both for muscle and non-muscle systems.

Materials and methods

Initial steps

Whole pig brains were obtained from the local slaughter house within 1–2 h after death. For each preparation, 8–10 brains were used which, after cleaning and removal of most of the white matter, gave ~500 g of the starting material composed mainly of cerebrum cortex. Particular attention was paid to the removal of blood vessels thus preventing contamination of the future extract by smooth muscle actomyosin. All subsequent steps were carried out at 4°C.

Cleaned brains were homogenized first in a Sorvall Omnimixer then in a glass-Teflon homogenizer in at least three volumes of a wash solution [containing 40 mM KCl, 1 mM MgCl₂, 10 mM imidazole, pH 6.8, 0.5 mM dithiothreitol (DTT)] with 1% Triton X-100 added and the homogenate was centrifuged for 1 h at 12 000 r.p.m. (Sorvall GSA rotor). The washing procedure was repeated at least three times with the exception that Triton X-100 was not included in the last two washes and the last centrifugation was carried out at 19 000 r.p.m. (Beckman 19 rotor).

Actomyosin

The residue obtained after the washing steps was quickly resuspended and homogenized in a low ionic strength actomyosin extraction solution (LAMES; Sobieszek and Small, 1976) containing 10 mM ATP (Na), 1 mM EDTA, 2 mM EGTA, 1 mM DTT, 60 mM KCl and 40 mM imidazole, pH 7.1. The insoluble material was promptly removed by 1 h centrifugation at 19 000 r.p.m. (Beckman 19 rotor) and the supernatant subjected to ammonium sulphate fractionation. It was found best to carry out two initial fractionations, the first between 0 and 20% and the second between 20 and 35% saturation, respectively, with two centrifugations at each step of 30 min at 12 000 r.p.m. (Sorvall rotor). Actomyosin precipitated between 35 and 55% ammonium sulphate saturation was dissolved in a myosin column solution, containing 0.6 M KCl, 11 mM MgCl₂, 10 mM ATP (Na), 1 mM EGTA, 0.5 mM DTT and 10 mM imidazole, pH 7.6 and clarified by 1 h centrifugation at 40 000 r.p.m. (Beckman 50 Ti rotor). The clear supernatant was then dialysed against the wash solution containing additionally 50 mM MgCl₂ and the precipitated crude actomyosin collected by centrifugation. This actomyosin was further purified by its resuspension in a small volume of wash solution and precipitation at 25% ammonium sulphate saturation.

Myosin

Pig brain myosin can be purified directly from the 45–55% ammonium sulphate fraction of the LAMES extract. This fraction, after solubilization in myosin column solution and clarification as for actomyosin, was directly applied onto a Sepharose 4B-CL column equilibrated with the same buffer. The first peak contained myosin and the second, actin and other impurities.

Alternatively, myosin was purified directly from actomyosin by solubilizing actomyosin in myosin column solution and applying on the same gel filtration column. In this case the second peak contained only actin and its associated proteins (see Results). Myosin purified by this second method was superior with respect to its activation by actin and level of phosphorylation (see Results) and was therefore used for most of the experiments described.

Tropomyosin

Pig brain tropomyosin was purified from two initial Triton X-100 washes.

After addition of solid KCl to 1 M, the pooled washes were heated to ~90°C, and, after cooling on ice, the precipitated proteins removed by centrifugation. By addition of solid ammonium sulphate up to 40% saturation most of the impurities could be removed and relatively pure tropomyosin could then be collected between 40 and 55% ammonium sulphate saturation. The tropomyosin pellet was then dissolved in wash solution and the tropomyosin collected again by isoelectric precipitation at pH 4.6.

Other proteins and procedures

Myosin light chain kinase activity was measured using purified gizzard myosin 20 000 dalton light chain as described previously (Sobieszek and Barylko, 1981). The purification of the myosin light chain kinase, the gizzard myosin light chains as well as their labelling with ³²P (used for monitoring myosin light chain phosphatase activity) has also been described in detail elsewhere (Sobieszek and Barylko, 1981). Skeletal muscle actin was prepared essentially by the method of Spudich and Watt (1971).

SDS-polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn (1969) with modifications according to Sobieszek and Bremel (1975). Protein concentration was estimated by the Biuret method using a protein standard from Sigma Corporation for calibration, or by the Biorad method.

Enzyme assays

The actin-activated ATPase activity was carried out in a solution containing 60 mM KCl, 1 mM (Mg) ATP, 1 mM MgCl₂, 1 mM DTT, 40 mM imidazole, pH 7.0, with 0.1 mM CaCl₂ or 2 mM EGTA added at 37°C. The reaction was initiated by addition of (Mg) ATP, terminated after 5–10 min with SDS and the liberated inorganic phosphate measured according to Fiske and Subbarow (1925). K-EDTA and Ca-activated ATPase activities were measured under similar conditions except that 0.6 M KCl and 2 mM EDTA or 10 mM CaCl₂ was present. In this case the reaction was terminated by the addition of trichloroacetic acid.

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