PHOSPHORYLATION OF A LIGHT CHAIN COMPONENT OF MYOSIN FROM SMOOTH MUSCLE

N. FREARSON, B. W. W. FOCANT* and S. V. PERRY
Department of Biochemistry, University of Birmingham, Birmingham, England

Received 12 December 1975
Original figures received 23 January 1976

1. Introduction

It has been demonstrated that the 18 000 dalton light chain component of myosin isolated from white skeletal muscle, sometimes called the DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] light chain is phosphorylated at one serine site [1] by a highly specific enzyme, myosin light chain kinase [2] present in muscle sarcoplasm and often associated with purified myosin preparations. A similar or possibly identical enzyme that phosphorylates the 19 000 dalton light chain component of myosin from cardiac and red skeletal muscles has also been shown to be present in these tissues [3]. Adelstein et al. [4] have described an enzyme that phosphorylates the 20 000 dalton light chain of platelet myosin which appears to be similar to muscle myosin light chain kinase although the platelet enzyme does not require Ca^{2+}, whereas the muscle enzyme requires low concentrations of this cation for activity [2].

We have postulated that a light chain of molecular weight 18 000 to 20 000, which we designate the 'P light chain', that can be phosphorylated by an endogenous light chain kinase, is a feature of myosin present in all vertebrate muscles [3]. This view is further supported by evidence presented in this communication that the 20 000 dalton light chain present in myosin from vertebrate smooth muscle is phosphorylated by an enzyme present in smooth muscle. The 20 000 dalton light chain component of smooth muscle myosin is also phosphorylated by the myosin light chain kinase of white skeletal muscle of the rabbit.

2. Materials and methods

Myosin: Myosins were prepared from the carotid and the muscular layer of the second stomach of the cow by the method of Hamoir and Gaspar-Godfroid [6]. Actomyosin was prepared from rabbit myometrium by the method of Needham and Cawkwell [7] modified by the inclusion of 0.1 mM EGTA in the original extraction medium. Myosin was isolated from the actomyosin dissolved in 0.6 M KCl, 40 mM Tris–32 mM HCl, pH 7.3, 5 mM $MgCl_2$, 2 mM ATP by ultracentrifugation to remove the actin [8]. Myosin was prepared from scallop (*Pecten maximus*) adductor muscles by the method of Szent-Gyorgyi et al. [9], and from the mussel (*Mytilus edulis* L.) adductor muscle by the same method as was used for rabbit myometrium.

Myosin light chain fraction: The whole light chain fraction was isolated from rabbit white skeletal and scallop adductor muscle myosins by the method of Perrie et al. [1] and from carotid and stomach myosin by the method of Leger and Focant [10]. The whole light chain fraction was phosphorylated by incubation with myosin light chain kinase as described by Morgan and Perry [11].

Sarcoplasmic extracts: Crude sarcoplasmic extracts were obtained from rabbit psoas muscle and rabbit uterus muscle by homogenisation of the fresh tissues

* Permanent Address: Laboratory of General Biology, University of Liege, Institut Van Beneden, 22, Quai Van Beneden, B-4020 Liege, Belgium.

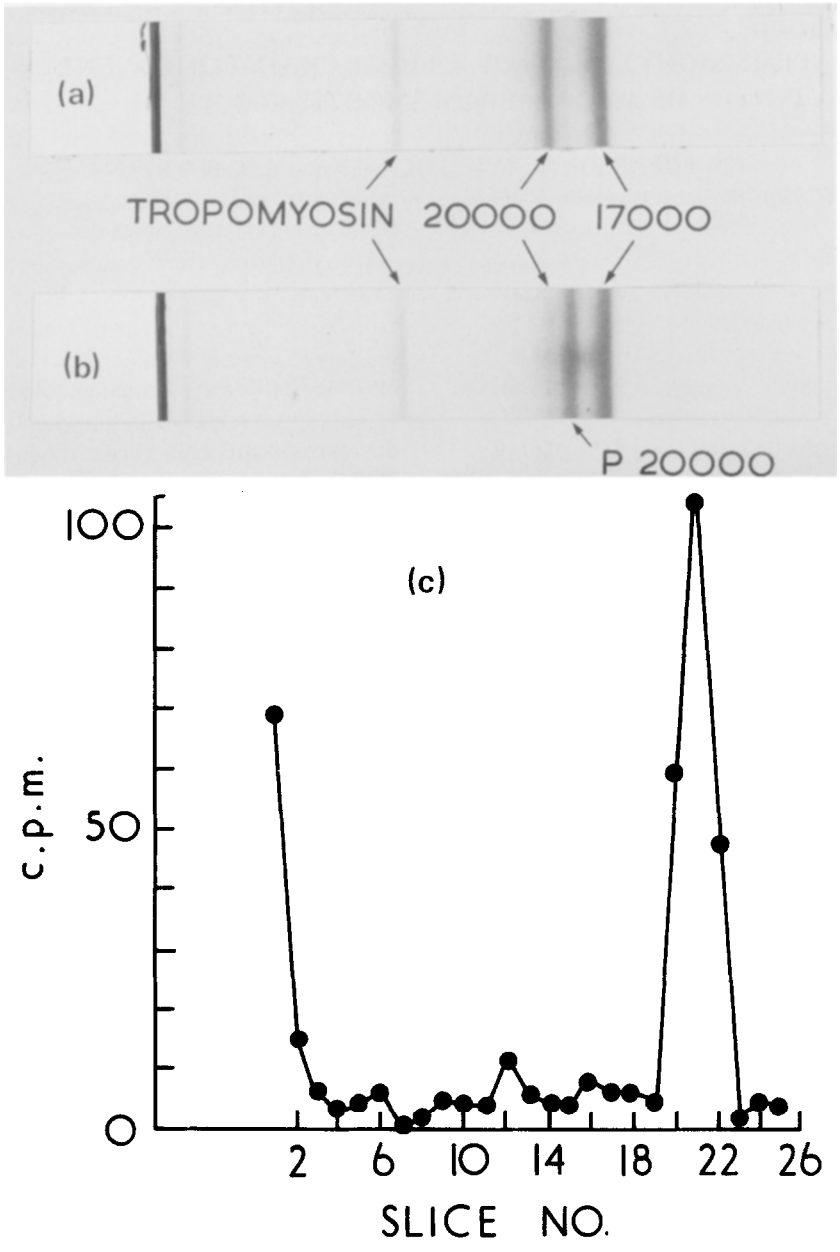

Fig.1. Electrophoresis of bovine stomach myosin phosphorylated with [γ-^{32}P]ATP. Myosin (8 mg/ml) incubated with [γ-^{32}P]ATP under standard assay conditions with myosin light chain kinase from rabbit white skeletal muscle (about 50 μg/ml) for 10 min at 30°C. Reaction stopped with solid urea and 400 μg of phosphorylated myosin applied to 8% polyacrylamide gel for electrophoresis. Control incubation carried out without ATP and added kinase. Light chains indicated by molecular weights; P-20 000, phosphorylated 20 000 dalton light chain. (a) Control incubation, all additions less [γ-^{32}P]ATP; (b) incubation with [γ-^{32}P]ATP; (c) distribution of radioactivity along the gel. Radioactivity estimated in 2 mm strips of gel.

in 5 volumes of 4 mM EDTA, 15 mM 2-mercaptoethanol, pH 7.0, followed by centrifugation at 2000 *g* for 35 min. The supernatant contained all the kinase and most of the phosphatase activity. These crude enzyme preparations were used within three days, during which time they showed little loss of activity.

Electrophoresis: Electrophoresis was performed on vertical slabs of 8% polyacrylamide using 20 mM Tris–122 mM glycine buffer, pH 8.6, containing 8 M urea [12].

Rabbit myosin light chain kinase: Preparations of rabbit white skeletal myosin light chain kinase were obtained by extraction of freeze-dried white skeletal myosin with 4 mM EDTA, pH 7.0 [2]. Putative myosin light chain kinase was extracted from scallop and *mytilus* adductor muscles by homogenisation in ten volumes of 40 mM KCl, 0.1 mM EDTA, 15 mM 2-mercaptoethanol, pH 6.5. Supernatants obtained after centrifugation at 2000 rev/min for 30 min were assayed for kinase activity.

Myosin light chain kinase activity was determined by incubating the whole light chain fraction of myosin with enzyme in 25 mM Tris–20 mM HCl, pH 7.6, 12.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM $CaCl_2$, 120 mM KCl. The reaction was initiated by addition of [γ-^{32}P]ATP (5 mM, approx. 1 μCi/μmole) and terminated by addition of solid urea (approx. 0.5 g/ml) or 5% trichloroacetic acid. Samples in urea were applied directly to polyacrylamide gels for electrophoresis. Protein precipitates and sliced gels were prepared for Cerenkov counting as described previously [13,14].

Myosin light chain phosphatase activity was assayed by incubation of ^{32}P-labelled whole light chain fraction of rabbit white skeletal muscle myosin with enzyme in 25 mM Tris–20 mM HCl, pH 7.6, 12.5 mM $MgCl_2$, 1 mM dithiothreitol 0.1 mM $CaCl_2$. The reaction was initiated by the addition of the ^{32}P-labelled light chain fraction (to final concentration of 0.4 mg/ml, approx. 1 μCi/μmole), and terminated by addition of five volumes of cold 5% trichloroacetic acid. The ^{32}P-phosphate released was estimated by Cerenkov counting in the protein-free supernatant obtained after centrifugation.

3. Results

On examination by polyacrylamide gel electrophoresis in 8 M urea at pH 8.6, preparations of myosin from cow carotid, cow stomach and rabbit uterus gave similar results (fig.1.). The light chains migrated as two main bands (corresponding to the 17 000 and 20 000 dalton light chain components, [10]), sometimes accompanied by minor 'satellite' bands [1,3].

3.1. *Phosphorylation of vertebrate smooth muscle myosin*

When each of the smooth muscle myosin samples was incubated with myosin light chain kinase from rabbit white skeletal muscle and [γ-^{32}P]ATP at 30°C for 10 min, under the conditions described in Materials and methods, a new band appeared, migrating faster than the 20 000 dalton component (fig.1b.). The newly-formed band was shown to be labelled with ^{32}P by sectioning the gel and measuring radioactivity in the slices (fig.1c).

Formation of the new band on electrophoresis and incorporation of ^{32}P was usually complete in about 10 min (figs.2 and 3) even in the absence of added kinase from rabbit white skeletal muscle, although the initial rates of phosphorylation were somewhat faster with added enzyme. If incubation was prolonged beyond about 10 min there was a progressive dephosphorylation shown by loss of ^{32}P and reappearance of the 20 000 dalton band of slower electrophoretic mobility. Dephosphorylation was faster in the absence of added rabbit enzyme. When all of the light chain

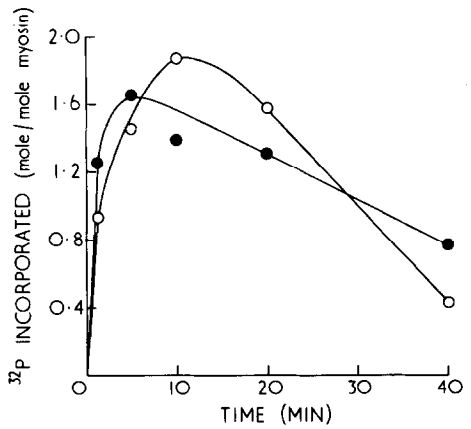

Fig.2. Phosphorylation of myosin from bovine stomach muscle. Myosin 8 mg/ml incubated at 30°C under standard myosin light chain kinase assay conditions. (○) No added enzyme; (●) myosin light chain kinase from rabbit skeletal muscle added (50 μg/ml).

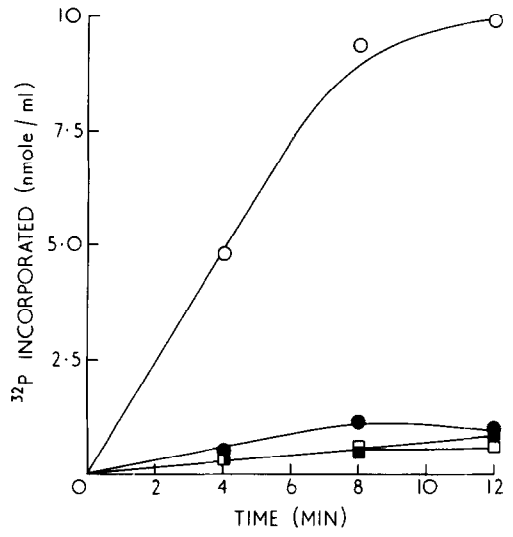

Fig.3. Incorporation of ^{32}P in the whole light chain fractions of myosin from rabbit uterus and rabbit white skeletal muscles. Light chains (12.5 mg/ml) were incubated under standard conditions with sarcoplasmic extracts of rabbit uterus and white skeletal muscles as indicated. (○) White muscle kinase and light chains; (●) smooth muscle kinase, white muscle light chains; (□) smooth muscle kinase and light chains; (■) white muscle kinase, smooth muscle light chains.

appeared converted into the phosphorylated form the amount of radioactivity incorporated corresponded to approx. 0.8–1.0 mole P/mole of light chain (assuming the mol. wt. of smooth muscle myosin was 470 000, and that it contains 2 mol of 20 000 dalton light chain per molecule [10]).

All the purified vertebrate smooth muscle myosins isolated contained intrinsic kinase activity for when they were incubated with ATP under the standard assay conditions (see Materials and methods) formation of the phosphorylated form of the 20 000 dalton light chain component could be shown either by gel electrophoresis or by the incorporation of ^{32}P.

The spontaneous dephosphorylation that occurred in such preparations of myosin suggested the presence of a phosphatase similar to the specific enzyme, myosin light chain phosphatase present in myosin prepared from rabbit white muscle [11,15].

3.2. *Phosphorylation of the light chain fraction of smooth muscle myosin*

Unlike the myosin of bovine stomach and carotids or rabbit uterus, the whole light chain fractions isolated from these proteins had no intrinsic kinase activity. Addition of myosin light chain kinase prepared from rabbit psoas muscle or rabbit uterus muscle, to the whole light chain fraction of rabbit uterus myosin resulted in phosphorylation (fig.3). The psoas muscle extract was about 12 times more active than the uterus extract with whole light chain fraction from myosin of rabbit white muscle as substrate. Both extracts had about the same activity towards the light chain fraction of uterus myosin.

3.3. *Kinase and phosphatase activities of sarcoplasm*

When smooth or striated muscle was homogenised in 4 mM EDTA all the kinase and phosphatase activities were present in the supernatant obtained after centrifugation. The sedimented material possessed negligible kinase or phosphatase activities when resuspended in 4 mM EDTA. These studies indicated that the kinase present per gram of white skeletal muscle was about 12 times more active than that present in a similar amount of smooth muscle in phosphorylating the light chain fraction from myosin of white skeletal muscle (table 1). In contrast the light chain phosphatase activities of the two muscles were similar. The phosphatase activities of sarcoplasmic extracts of striated and smooth muscle were 90% inhibited by 50 mM NaF. Samples of bovine smooth muscle myosins that had been stored for several weeks in 50% glycerol at −30°C still contained appreciable kinase and phosphatase activities.

Table 1
Myosin light chain kinase and phosphatase activities of rabbit muscle

	Kinase (nmoles/g/min)	Phosphatase (nmoles/g/min)
Fast white (psoas)	456 ± 28 (4)	46 ± 4.0 (6)
Slow red (soleus)	295 ± 74 (4)	63.6 ± 4.5 (6)
Cardiac	16.5 ± 3.5 (4)	55 ± 5.0 (6)
Smooth (uterus)	38 ± 3.6 (4)	62 ± 4.7 (6)

Results given as means ± standard deviation. Number of duplicate assays given in brackets. Sarcoplasmic extracts prepared and assayed as described in Materials and methods section. For kinase assay 10 μl extract incubated in total vol. of 100 μl; phosphatase assay 0.1 ml incubated in total vol. of 1 ml.

Up to 80% of the intrinsic kinase activity of rabbit white skeletal muscle myosin can be removed by three successive washings with 4 mM EDTA, pH 7.0 (N. Frearson and M. Morgan unpublished observations); similar treatment of rabbit uterus myosin left the intrinsic kinase activity unaffected.

3.4. *Phosphorylation of invertebrate smooth muscle myosin*

When preparations of myosin made from *Pecten maximus* and the whole light chain fraction isolated from it were incubated with [γ-^{32}P]ATP under standard myosin light chain kinase assay conditions, no incorporation of phosphate could be detected in either the light or heavy chain fraction of the myosin. On addition of a preparation of myosin light chain kinase from rabbit white muscle to the system there was a low level of incorporation which was 3–4% of that obtained with myosin from rabbit white skeletal muscle or the whole light chain fraction isolated from it, as substrate. No obvious change in mobility of either of the scallop myosin light chains could be detected on electrophoresis in 8 M urea, pH 8.6, after incubation. Similar studies with the myosin from posterior adductor muscle of mytilus showed low incorporation of radioactivity when incubated with [γ-^{32}P]ATP and putative *mytilus* kinase. In this case most of the radioactivity appeared to be localized in the heavy chain fraction of the myosin.

Some myosin light chain kinase activity could be detected in extracts of scallop adductor muscle. Attempts to purify the kinase were unsuccessful as the low levels of activity were rapidly lost. The crude enzyme, however, did catalyse the incorporation of ^{32}P into the whole light chain fractions of myosin from rabbit white skeletal muscle and pecten adductor muscle. The rates were roughly similar for the two substrates but much lower than obtained under the same conditions with the light chain fraction from rabbit white muscle myosin as substrate and using the enzyme isolated from rabbit white skeletal muscle (fig.4).

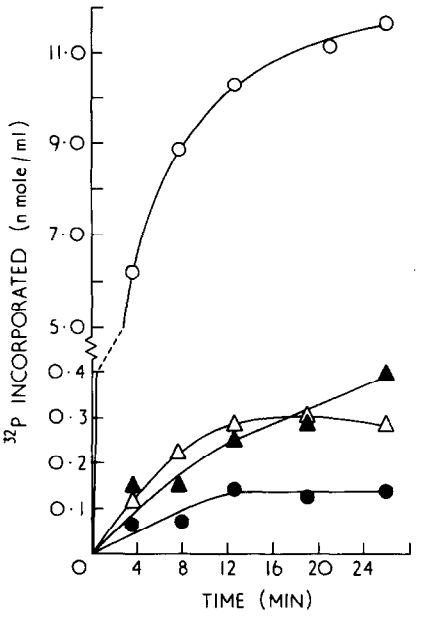

Fig.4. Incorporation of ^{32}P in the whole light chain fractions of myosin from pecten adductor and rabbit white skeletal muscles. Licht chains (10 mg/ml) were incubated under standard conditions with kinase preparations indicated. (○) Rabbit skeletal light chains and kinase; (△) scallop adductor light chains and kinase; (●) rabbit skeletal light chains, scallop adductor kinase; (▲) scallop adductor light chains, rabbit skeletal kinase.

4. Discussion

The findings with the myosin of vertebrate smooth muscle further confirm the view [3,15] that a light chain of mol. wt. 18 000–20 000 that can be phosphorylated by myosin light chain kinase is a feature of myosins from vertebrate muscles. In each case phosphorylation leads to a change in electrophoretic mobility at pH 8.6. This light chain we have designated the 'P light chain'.

In the case of myosin from invertebrate adductor muscle the evidence for phosphorylation is much less definite. Some incorporation was obtained with myosin from the scallop and there was evidence of the presence of an endogenous kinase. The activity of the endogenous light chain kinase was very low compared to that of white skeletal muscle and the whole light chain fraction isolation from scallop adductor myosin was a poor substrate for the kinase prepared from rabbit white skeletal muscle.

In no case did the extent of incorporation in adductor myosin permit clear localization of ^{32}P in a light chain band or the demonstration of a change in

mobility due to phosphorylation. The evidence suggests that phosphorylation of myosin light chains is not a significant event in scallop adductor muscle. If it is the enzymes involved are highly specific and possibly present in very low amounts or are too unstable to permit study in homogenates without special precautions.

Myosin light chain kinase is mainly present in the sarcoplasm in both striated and smooth muscles although it appears to be less readily extracted from smooth muscle myosin with which it associates during preparation.

Whereas the amount of myosin light chain kinase per gram of tissue are very much higher in skeletal muscle than in smooth, the light chain phosphatase level was very similar in all muscle types. Therefore, in view of the low amounts of myosin per gram of smooth muscle, there is relatively more phosphatase activity per myosin molecule in the latter tissue. Neglecting any special mechanisms that may be involved in their regulation, the relative levels of the phosphorylating and de-phosphorylating enzymes suggest that the 'P light chain' would be more extensively phosphorylated in white skeletal than in smooth muscle.

Acknowledgements

This work was supported in part by a Research Grant from the Medical Research Council. B.W.W. Focant is 'chercheur qualifie' au Fonds National de la Recherche Scientifique of Belgium and was supported by an EMBO short-term fellowship.

References

[1] Perrie, W. T., Smillie, L. B. and Perry, S. V. (1973) Biochem. J. 135, 151–164.
[2] Pires, E., Perry, S. V. and Thomas, M. A. W. (1974) FEBS Lett. 41, 292–296.
[3] Frearson, N. and Perry, S. V. (1975) Biochem. J. 151, 99–107.
[4] Adelstein, R. S., Conti, M. A. and Anderson, W. (1973) Proc. Nat. Acad. Sci., USA 70, 3115–3119.
[5] Adelstein, R. S. and Conti, M. A. (1975) Nature 256, 597–598.
[6] Hamoir, G. and Gaspar-Godfroid, A. (1964) Angiologica 1, 317–337.
[7] Needham, D. M. and Cawkwell, J. M. (1956) Biochem. J. 63, 337–344.
[8] Weber, A. (1956) Biochim. Biophys. Acta 19, 345–351.
[9] Szent-Gyorgyi, A. G., Cohen, C. and Kendrick-Jones, J. (1971) J. Mol. Biol. 56, 239–258.
[10] Leger, J. J. and Focant, B. W. W. (1973) Biochim. Biophys. Acta 328, 166–172.
[11] Morgan, M. and Perry, S. V. (1975) Biochem. J., in the press.
[12] Perrie, W. T. and Perry, S. V. (1970) Biochem. J. 119, 31–38.
[13] Perry, S. V. and Cole, H. A. (1973) Biochem. J. 131, 425–428.
[14] Perry, S. V. and Cole, H. A. (1974) Biochem. J. 141, 733–743.
[15] Perry, S. V., Cole, H. A., Morgan, M., Moir, A. J. G. and Pires, E. (1975) Proc. 9th FEBS Meeting, Vol. 31, 168–176.