Biochem. J. (1977) **167**, 137–146
Printed in Great Britain

Purification and Properties of Myosin Light-Chain Kinase from Fast Skeletal Muscle

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(*Received* 18 *February* 1977)

1. A procedure is described for the isolation of myosin light-chain kinase from rabbit fast skeletal muscle as a homogeneous protein. 2. Myosin light-chain kinase is a monomeric enzyme of mol.wt. 77000. Under some conditions of storage it is converted into components of mol.wts. about 50000 and 30000 that possess enzymic activity. 3. The enzyme is clearly different in structure and properties from any other protein kinase so far isolated from muscle. 4. The enzyme is highly specific for the P-light chain (18000–20000-dalton light chain) of myosin and requires Ca^{2+} for activity. 5. The P-light chain is phosphorylated at a similar rate whether isolated or associated with the rest of the myosin molecule. 6. The effects of pH, bivalent cation and other nucleotides on the enzymic activity are described. 7. The role of the phosphorylation of the P-light chain of myosin in muscle function is discussed.

The original reports (Perrie *et al.*, 1972, 1973) that the 18000-dalton light-chain component of myosin from rabbit white skeletal muscle, the P-light chain (Frearson & Perry, 1975), could exist in phosphorylated and dephosphorylated forms implied that enzyme systems were present in muscle for their interconversion. Although impure preparations of phosphorylase kinase and 3′:5′-cyclic AMP-dependent protein kinase would slowly catalyse the phosphorylation of the P-light chain of myosin, the ability of these enzyme preparations to catalyse the phosphorylation of the P-light chain was lost on further purification (Pires *et al.*, 1974). This suggested that myosin-phosphorylating activity was due to contamination with a new protein kinase not identical either with phosphorylase kinase or with 3′:5′-cyclic AMP-dependent protein kinase. The enzyme was therefore named myosin light-chain kinase, partially purified, and some properties were briefly described (Pires *et al.*, 1974). The highly specific nature of the kinase and its widespread distribution in white and red skeletal, cardiac and smooth muscles (Frearson & Perry, 1975; Frearson *et al.*, 1976*a*) imply that the phosphorylation of the P-light chain is of special significance for muscle function. Further support for a functional role for the phosphorylation of the P-light chain of myosin has been provided by the report of the purification and characterization of myosin light-chain phosphatase (Morgan *et al.*, 1976). This enzyme has a similar specificity and localization in muscle to myosin light-chain kinase, is not identical with any known protein phosphatase and has a mol.wt. of about 70000.

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The present report describes the isolation of myosin light-chain kinase from muscle as a homogeneous protein of mol.wt. 77000 and an investigation of its properties. Some aspects of the work have been briefly reported elsewhere (Perry *et al.*, 1975, 1976).

Materials and Methods

Materials

Dithiothreitol, Tris, 3′:5′-cyclic AMP, disodium salts of AMP and ADP, β-galactosidase, phosphorylase *b*, bovine serum albumin, ovalbumin and histone (calf thymus type III-S) were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. The disodium salt of ATP was from Kyowo Makko Kogyo Co., Tokyo, Japan. [γ-^{32}P]-ATP was from The Radiochemical Centre, Amersham, Bucks., U.K. CNBr was from R. Emanuel, London W.5, U.K. DEAE-cellulose was from Whatman Biochemicals, Maidstone, Kent, U.K. Sephadex G-200 was from Pharmacia (G.B.), London W.5, U.K. Ultrogel AcA44 was from LKB Instruments, South Croydon, Surrey CR2 9PX, U.K. Acrylamide and *NN′*-methylenebisacrylamide were from Serva Feinbiochemica, D-69 Heidelberg 1, West Germany. Ampholine carrier (40%) was from LKB Products AB, Stockholm, Bromma 1, Sweden. Coomassie Brilliant Blue R was from George T. Gurr, High Wycombe, Bucks., U.K. All the other chemicals mentioned in this work were either from BDH Chemicals, Poole, Dorset, U.K., or from Fisons, Loughborough, Leics., U.K.

Preparation of dephosphorylated myosin

Myosin in which the P-light chain was completely dephosphorylated was prepared as described by

Pires *et al.* (1974) from white skeletal muscle of the rabbit. Unless otherwise stated all protein preparations were carried out at 4°C.

For crude preparations of myosin light-chain kinase, twice-precipitated myosin suspended in 0.05M-KCl was freeze-dried.

Preparation of the light-chain fraction of myosin

The whole light-chain fraction of myosin, consisting of the P-light chain and the alkali light chains (Weeds & Frank, 1972), was isolated from dephosphorylated myosin by the method described by Perrie & Perry (1970). About 1g of whole light-chain fraction was isolated from 1kg of muscle by this procedure and freeze-dried for storage. Before use 600mg of freeze-dried light-chain fraction was dissolved in 50ml of water, dialysed against 10 litres of water containing 10mM-2-mercaptoethanol to remove traces of salt and guanidine, and again freeze-dried.

The P-light chain was isolated from whole light-chain fraction by the method of Perrie *et al.* (1973), with the modification that the ethanol fractionation was carried out at pH6.1, rather than at pH6.5 as in the original method, to improve the separation.

Myosin light-chain kinase assays

The P-light-chain content of the whole light-chain fraction used as substrate was determined by densitometric scanning of gels obtained by polyacrylamide-gel electrophoresis of the whole fraction in sodium dodecyl sulphate, pH7.0 (see below). Estimates of the P-light-chain content varied from 40 to 50%, with an average value of 45%, which was the value used as a routine, except when an actual determination was carried out on the preparation.

The standard reaction mixtures (total volume 0.1–0.5ml) contained 50mM-Tris/40mM-HCl, pH7.6, 12.5mM-magnesium acetate, 0.1mM-$CaCl_2$, 1mM-dithiothreitol, 5.5mM-[γ-^{32}P]ATP (approx. 2μCi/μmol at day zero) and 15mg of whole light-chain fraction of rabbit white-skeletal-muscle myosin/ml. The reaction was carried out at 25°C and was usually started by addition of the [γ-^{32}P]ATP and stopped by pipetting a portion, usually 0.1ml, into 2.5ml of cold 5% (w/v) trichloroacetic acid containing 5mM-ATP. The protein precipitate obtained was washed with 3×2.5ml of cold 5% trichloroacetic acid containing 1mM-ATP, resuspended in 2.5ml of 0.1M-NaOH and the volume made up to 10ml with water. The ^{32}P incorporated into the protein was measured by the Čerenkov method (Gould *et al.*, 1972) by using a Philips scintillation counter model PW 4540.

Preparation of myofibrils

Myofibrils were prepared from rabbit white skeletal muscle by the method of Perry & Zydowo (1959).

ATPase assays*

ATPase assays were carried out in a final volume of 2ml containing 50mM-Tris (adjusted to pH7.6 with 12M-HCl), 5.5mM-ATP, either 12.5mM- or 6.25mM-magnesium acetate and 1mM-dithiothreitol under the conditions described by Schaub & Perry (1969).

Ca^{2+} buffers

Ca^{2+} EGTA buffers were prepared by using the following equation:

$$[Ca^{2+}]_{total} = \frac{[Ca^{2+}]_{free}(K_a[EGTA] + K_a[Ca^{2+}]_{free} + 1)}{K_a[Ca^{2+}]_{free} + 1}$$

The association constant (K_a) was taken as $3.7 \times 10^7 M^{-1}$ at pH7.5 (Charberek & Martell, 1959).

Protein determination

Protein was assayed by the method of Lowry *et al.* (1951) or by a micro-method involving precipitation by tannin (Mejbaum-Katzenellenbogen & Dobryszycka, 1959).

Preparation of hydroxyapatite

Hydroxyapatite was prepared as described by Bernardi (1971).

Preparation of P-light-chain–Sepharose 4B affinity column

Sepharose 4B (60g) was activated by the method of Porath *et al.* (1967). Dephosphorylated P-light chain (200mg) was dissolved in 5ml of 0.1M-$NaHCO_3$ and added to the activated Sepharose 4B suspended in 60ml of 0.1M-$NaHCO_3$, and the reaction was left to proceed for 20h in the cold with gentle stirring. The P-light-chain–Sepharose 4B was washed by centrifugation three times for 5min at 2000*g*, each with 6vol. of 0.1M-$NaHCO_3$, and resuspended with 10mM-NaH_2PO_4, adjusted to pH6.5 with 1M-NaOH, for preparation of the column (2.5cm×12cm) consisting of approx. 50g wet wt. of P-light-chain–Sepharose 4B. Before use the column was washed with 3vol. of the 10mM-sodium phosphate buffer, pH6.5, and afterwards with the same buffer containing 0.5M-NaCl. No apparent change in properties was observed after ten cycles of use.

Gel electrophoresis

Gel electrophoresis in 6M-urea was in general carried out on polyacrylamide slab gels as described by Perrie *et al.* (1973). The precise conditions are given in the text where appropriate. All the gels were stained as described by Perrie *et al.* (1973).

* Abbreviation: ATPase, adenosine triphosphatase.

For the isolation of bands with myosin light-chain kinase activity electrophoresis was carried out on 7.5% polyacrylamide slab gels in 25mM-Tris/80mM-glycine, pH8.6; 0.25mM-dithiothreitol was added to the running buffers. Electrophoresis was carried out in the cold-room (0–4°C), usually until the marker dye, Bromophenol Blue, reached the bottom of the slab. Thin vertical strips from the centre and from each side of the slab were cut and stained to locate the protein bands. The remaining unstained portions of the gels were sliced in 2mm horizontal strips and the protein was eluted by suspension for 4–12h into 1.2ml of 25mM-Tris/2mM-EDTA (adjusted to pH7.8 with 12M-HCl)/10mM-NaCl/0.25mM-dithiothreitol. The activity was assayed in 10μl samples of the supernatant obtained after centrifuging (10min at 2000*g*) down the polyacrylamide, as described above.

Electrophoresis in sodium dodecyl sulphate was carried out in 0.1M-sodium phosphate buffer, pH7.0, as described by Weber & Osborn (1969) or in 85mM-Tris/400mM-boric acid buffer, pH7.0, described by Perrie *et al.* (1973). Before electrophoresis samples were incubated for 5min at 100°C in double-concentration running buffer containing 1% 2-mercaptoethanol and 8M-urea. For molecular-weight determinations phosphorylase A (mol.wt. 92500) and the protein standards listed below for gel filtration were used.

Isoelectric focusing

Isoelectric focusing in polyacrylamide gels was carried out by the methods of Righetti & Drysdale (1971) and Vesterberg (1972). The gels were 8.5cm long in 10cm×0.5cm tubes and contained 4% polyacrylamide (97.5% acrylamide, 2.5% bisacrylamide) and 2% (w/v) ampholyte carrier, pH range 3–10. Polymerization was achieved by addition of 0.05ml of *NNN'N'*-tetramethylethylenediamine and 0.1ml of 10% (w/v) ammonium persulphate to 19.7ml of the polyacrylamide/ampholyte solution. Samples (0.5ml) containing about 300μg of protein and 2% ampholyte were titrated to pH9.0 with 0.1M-NaOH. The upper electrode vessel was filled with 0.02M-NaOH and the lower one with 10mM-H_3PO_4. After 10min of electrophoresis at 1mA per tube the samples (10–40μl) were applied. Electrofocusing was performed at a constant current of 1mA per tube until the voltage had risen to 200V, at which value it was maintained for 12h.

Conductivity

Conductivity measurements were carried out with a Radiometer conductivity-meter type CDM 2E.

Molecular-weight determinations by gel filtration

A column (1.5cm×80cm) of Ultrogel AcA44 was equilibrated with 12M-Tris/2mM-EDTA (pH adjusted to 7.6 with 12M-HCl)/100mM-NaCl/0.25mM-dithiothreitol and calibrated with transferrin (mol.wt. 77000), bovine serum albumin (mol.wt. 68000) and ovalbumin (mol.wt. 45000). For molecular-weight determination a 0.3ml sample containing 2mg was applied to the column and elution carried out at a flow rate of about 9ml/h. The void volume, V_0 (Andrews, 1965), was determined with Blue Dextran.

Purification of myosin light-chain kinase

Step 1. New Zealand White rabbits were killed by a blow on the back of the neck, exsanguinated and rapidly skinned. The back and hind-leg muscles were quickly dissected out and placed on ice. Minced muscle (600g) was extracted by gentle stirring for 30min with 1800ml of 10mM-NaH_2PO_4/4mM-EDTA (adjusted to pH6.5 with 1M-NaOH)/0.25mM-dithiothreitol. The homogenate was then centrifuged at 2000*g* for 20min and the supernatant fluid collected and filtered through glass wool.

Step 2. The extract (about 1800ml) was applied to a DEAE-cellulose column (4.5cm×11.5cm) that had been equilibrated with the buffer used for extraction of the muscle (see under 'Step 1'). The column was washed with 300ml of the buffer and the kinase was then eluted with 300ml of extraction buffer containing 0.15M-NaCl.

Step 3. The solution eluted from DEAE-cellulose by the extraction buffer containing 0.15M-NaCl (about 300ml) was diluted 5-fold with 25mM-Tris/2mM-EDTA (adjusted to pH7.6 with 12M-HCl)/0.25M-dithiothreitol and applied to a second DEAE-cellulose column (3.0cm×13.0cm) previously equilibrated against this buffer. The column was first washed with 300ml of the buffer used for equilibration and then developed with 600ml of the buffer in which a linear gradient of NaCl (0–0.2M) was produced by mixing chambers of 300ml capacity. The fractions (10ml) in the regions indicated in Fig. 1 were assayed for protein and for myosin light-chain kinase activity and the most active pooled. Steps 1–3 were completed within 24h.

Step 4. The pooled fraction (90–100ml, A_{280} 0.9–1.0) was adjusted to 55% saturation by addition of solid $(NH_4)_2SO_4$. Protein was allowed to precipitate for 15min and then collected by centrifugation at 10000*g* for 15min. The supernatant was discarded and the precipitate dissolved in 1.5ml of 25mM-Tris/2mM-EDTA (adjusted to pH7.6 with 12M-HCl)/100mM-NaCl/0.25mM-dithiothreitol.

Step 5. The solution (about 2ml, 45–55mg/ml) of the protein precipitated by $(NH_4)_2SO_4$ was dialysed for 2h against 25mM-Tris/2mM-EDTA (adjusted to pH7.6 with 12M-HCl)/100mM-NaCl/0.25mM-dithiothreitol and applied to an Ultrogel AcA44 column (2.5cm×85cm) previously equilibrated with the same buffer. Fractions (6ml) were collected on elution with

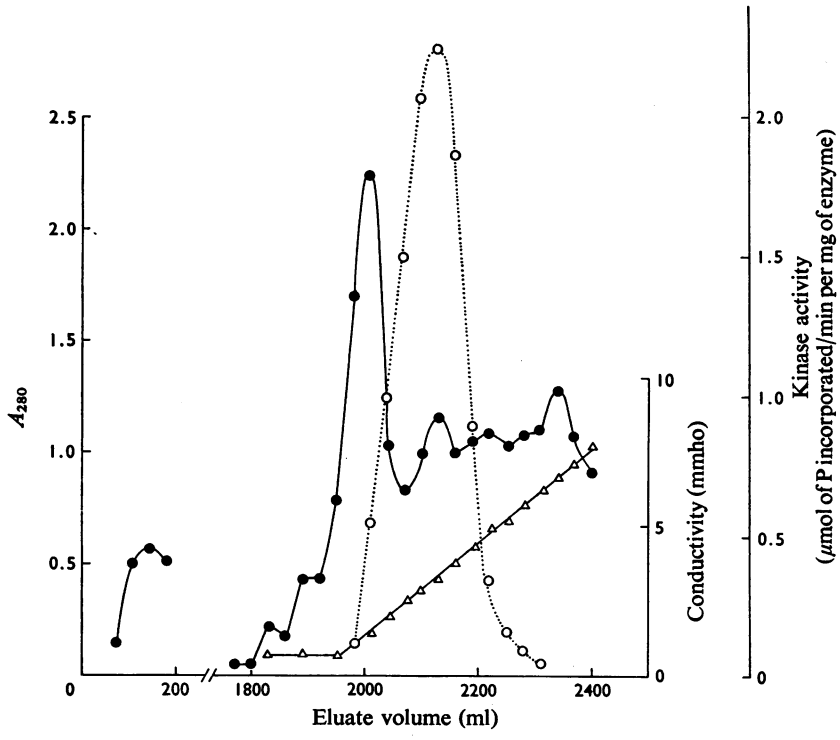

Fig. 1. *Chromatography on DEAE-cellulose of crude myosin light-chain kinase preparation*
A sample of crude myosin light-chain kinase (1500ml containing 900mg of protein), prepared as described under 'Step 3' (see the Materials and Methods section), was applied to a column (3cm×13cm) of DEAE-cellulose previously equilibrated with 25mM-Tris/2mM-EDTA (adjusted to pH7.6 with 12M-HCl)/0.25mM-dithiothreitol. The column was developed with the buffer containing a linear gradient of NaCl, and 10ml fractions were collected. ●, A_{280}; ○, myosin light-chain kinase activity; △, conductivity.

the same buffer and assayed for protein and for myosin light-chain kinase activity (Fig. 2).

Step 6. The most active fractions (see the Results section) obtained in the previous step were pooled together (18ml, A_{280} 0.5) and dialysed against 10mM-NaH_2PO_4 (adjusted to pH6.5 with 1M-NaOH)/5mM-magnesium acetate/0.25mM-dithiothreitol and applied to a P-light-chain–Sepharose 4B affinity column (2cm×12.5cm) previously equilibrated with the buffer against which the sample was dialysed. The column was washed with 3 bed vol. of buffer and the kinase eluted by application of 80ml of 0.1M-sodium phosphate buffer (pH6.5)/0.25mM-dithiothreitol.

Step 7. The protein peak obtained in the previous step (about 80ml, 0.05mg/ml) was dialysed for 7h against the 10mM-sodium phosphate buffer (adjusted to pH6.5 with 1M-NaOH)/5mM-magnesium acetate/0.25mM-dithiothreitol, diluted 4-fold with the same buffer and applied to a hydroxyapatite column (1.6cm×2.5cm). The material was eluted with 10ml of 0.15M-NaH_2PO_4 (adjusted to pH6.5 with 1M-NaOH)/0.25mM-dithiothreitol.

This procedure produced about 10ml of purified enzyme solution (0.28–0.42mg/ml). It was stored at −10°C after addition of solid sucrose to 10% (w/v).

Results

Purification of the enzyme

Although myosin preparations were invariably contaminated with light-chain kinase, even after several reprecipitations (Pires *et al.*, 1974), most of the enzyme was found in the protein fraction of muscle extracted at low ionic strength, i.e. in the sarcoplasmic fraction. On extraction of muscle mince with 3vol. of 10mM-NaH_2PO_4/4mM-EDTA/0.25mM-dithiothreitol, adjusted to pH6.5 with 1M-NaOH, about 60–80% of the total enzymic activity was present in the supernatant obtained on centrifugation for 20min at 2000*g*. Second and third extractions under similar conditions yielded 15–25% and about 5–10% of the total activity respectively. A relatively crude preparation of the enzyme with

EXPLANATION OF PLATE 1

Polyacrylamide-gel electrophoresis of purified myosin light-chain kinase preparations

Gels and buffers were: (*a*) and (*b*), 10% polyacrylamide, 0.1M-sodium phosphate (pH7.0), 1% sodium dodecyl sulphate; (*c*) 7.5% polyacrylamide, 20mM-Tris/125mM-glycine, pH8.6; (*e*)–(*k*), 7.5% polyacrylamide, 85mM-Tris/400mM-boric acid, pH7.0, 1% sodium dodecyl sulphate. Samples for (*f*)–(*h*) were prepared as follows: 0.5ml samples of eluate from each peak illustrated in Fig. 8 were freeze-dried, dissolved in the Tris/borate buffer containing 8M-urea and 1% 2-mercaptoethanol, incubated for 5min at 100°C and applied to the gel. O, Origin. (*a*) Approx. 40μg each of the standard proteins β-galactosidase (mol.wt. 140000), phosphorylase (90000), transferrin (77000), bovine serum albumin (68000) and ovalbumin (45000); (*b*) 30μg of purified kinase; (*c*) 30μg of purified kinase isoelectrically focused on 4% polyacrylamide gel containing 2% (w/v) ampholyte, pH range 3–10; (*e*) 20μg of protein standards as in (*a*), but ovalbumin omitted; (*f*) Peak I, Fig. 8; (*g*) Peak II, Fig. 8; (*h*) Peak III, Fig. 8; (*i*) 30μg of enzyme kept for 15 days at 2°C in 25mM-Tris/2mM-EDTA (adjusted to pH7.6 with 12M-HCl)/100mM-NaCl/0.25mM-dithiothreitol; (*j*) 30μg of enzyme (0.3mg/ml) kept for 15 days at 2°C in 0.15M-sodium phosphate buffer (pH6.8)/0.25mM-dithiothreitol; (*k*) 30μg of enzyme (0.3mg/ml) kept for 15 days at −10°C in 0.15M-sodium phosphate buffer (pH6.8)/0.25mM-dithiothreitol/10% sucrose.

specific activity about 5 times that of the initial extract obtained from whole muscle (step 1) could be obtained by extracting freeze-dried myosin with 40vol. (v/w) of 4mM-EDTA, adjusted to pH7.0 with 1M-Tris. In this way active extracts could be obtained even after storing freeze-dried myosin at −10°C for 3–4 months. Although this was a rapid and convenient method of preparing crude kinase preparations, the yields of enzyme per g of muscle were low, and freeze-dried myosin was abandoned as a source of crude enzyme for further purification.

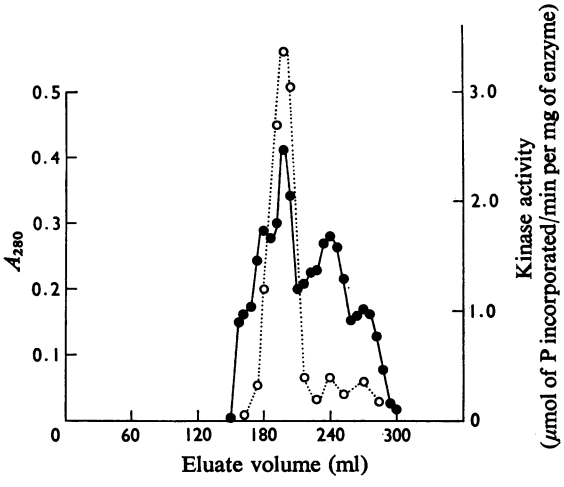

Fig. 2. *Gel filtration of partially purified myosin light-chain kinase*

A sample of myosin light-chain kinase (approx. 50mg in 1.8ml) prepared as described under 'Step 5' (see the Materials and Methods section) was applied to a column (2.5cm×85cm) of Ultrogel AcA44 equilibrated with 25mM-Tris/2mM-EDTA (adjusted to pH7.6 with 12mM-HCl)/0.25mM-dithiothreitol. Fractions (6ml) were collected on elution with the same buffer. ●, A_{280}; ○, myosin light-chain kinase activity.

The enzyme purified by the procedure described in the Materials and Methods section was homogeneous, and when assayed under standard conditions (see the Materials and Methods section) at 25°C had a specific activity of 25±5μmol incorporated into P-light chain/min per mg of protein. A summary of the purification achieved in each step is presented in Table 1.

Although chromatography on the P-light-chain–Sepharose 4B affinity column was a very effective procedure for purification when the enzyme was the major component of the preparation, it was less effective at early stages of the purification. Minor components present after the affinity-chromatography step, representing only a few percent of the total protein, were removed by the final hydroxyapatite step. The complete procedure yielded an enzyme preparation that migrated as a single band, representing at least 95% of the total protein, on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate at pH7.0 and at 8.6 under non-dissociating conditions (Plate 1*b* and 1*c*). When isoelectric focusing was carried out on the preparation, a major band, accompanied by a minor band estimated to represent less than 5% of the total protein, was obtained (Plate 1*d*).

Molecular weight

When the purified kinase was subjected to gel filtration on Ultrogel AcA44 in 25mM-Tris (adjusted to pH7.6 with 12M-HCl)/2mM-EDTA/0.3M-NaCl/0.25mM-dithiothreitol, the protein was eluted as a single symmetrical peak corresponding to a mol.wt. of approx. 77000. A similar value for the molecular weight was obtained by electrophoresis on polyacrylamide gel in 0.1% sodium dodecyl sulphate/0.1M-sodium phosphate buffer, pH7.0.

Myosin and the isolated light-chain fraction as substrates for myosin light-chain kinase

There are a number of advantages in using the isolated light-chain fraction rather than whole

Table 1. *Summary of purification achieved at various stages during the preparation of myosin light-chain kinase*

Results are from six preparations each starting with 600g of minced rabbit muscle. A unit of activity is 1μmol of P incorporated into P-light chains/min. N.D., Not determined.

Step	Fraction	Protein (mg)	Activity: Total (units)	Activity: Specific (units/mg of protein)	Purification (fold)	Yield (%)
1	Sarcoplasmic extract	20400–32400	833–873	0.025–0.031	—	—
2	1st DEAE-cellulose	780–980	628–722	0.625–0.911	20–29	79–82
3	2nd DEAE-cellulose	73–93	311–395	3.72–4.78	126–132	38–45
4	55%-Satd.-$(NH_4)SO_4$ pellet	45–55	N.D.	N.D.	N.D.	N.D.
5	Ultrogel AcA44	8.3–15.9	127–168	10.8–15.8	350–365	16–19
6	P-light-chain–Sepharose 4B	3.0–4.5	70–110	20.9–29.5	803–857	8–12
7	Hydroxyapatite	2.8–4.2	65–100	22.1–30.4	820–870	7–11

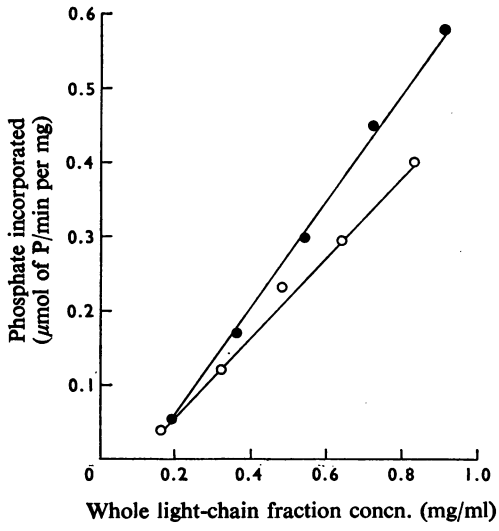

Fig. 3. *Comparison of rates of phosphorylation of P-light chain in myosin and in the isolated whole light-chain fraction*

Purified myosin light-chain kinase (1 μg/ml) was incubated with increasing concentrations of either dephosphorylated myosin or the whole light-chain fraction isolated from dephosphorylated myosin in otherwise standard assay conditions (see the Materials and Methods section). It was assumed that the light chains represented 14% of the myosin molecule by weight. Enzymic activity was determined by stopping the reactions with 1 vol. (v/v) of 10 M-urea, adding 0.2 ml of bovine serum albumin (5 mg/ml) per 0.1 ml of incubation mixture, precipitating the protein immediately with 10% (w/v) trichloroacetic acid containing 1 mM-ATP and then proceeding as indicated in the Materials and Methods section. ●, Myosin; ○, light-chain fraction.

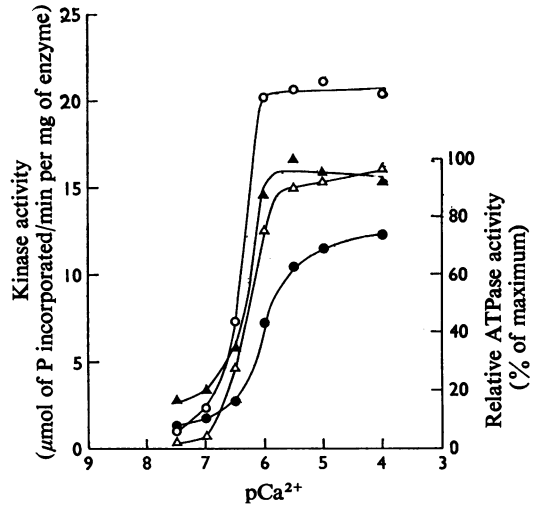

Fig. 4. *Effect of Ca^{2+} concentration on myosin light-chain kinase activity*

Purified myosin light-chain kinase (1 μg/ml) or myofibrils (0.25 mg/ml) were incubated in 50 mM-Tris (adjusted to pH 7.6 with 1 M-HCl)/5.5 mM-ATP, and light-chain fraction (15 μg/ml) as appropriate. The Ca^{2+} concentration was varied by using Ca^{2+}/EGTA buffers (see the Materials and Methods section). Myosin light-chain kinase activity and myofibrillar ATPase were determined as indicated in the Materials and Methods section: ATPase activity at pCa^{2+} 5.5 and 6.25 mM-$MgCl_2$ was taken at 100%. ○, △, Kinase activity; ●, ▲, ATPase activity; ○, ●, 12.5 mM-Mg^{2+}; △, ▲, 6.25 mM-Mg^{2+}.

myosin as substrate for the kinase in kinetic studies. In the first place, unlike myosin, which is not easily solubilized after freeze-drying, the light-chain fraction is readily stored and redissolved in buffer after this procedure. Further, as the P-light chain represents only about 7% by weight of the myosin, very high myosin concentrations, expressed in mg of protein/ml, were required for saturation of the enzyme. This led to difficulties in the precipitation and washing procedures involved in the measurement of covalently linked ^{32}P. When assayed under standard conditions at identical concentrations of P-light chain in the range 0.1–0.5 mg/ml, the rate of phosphorylation was slightly less with the isolated whole light-chain fraction than with myosin as the substrate (Fig. 3). It was uncertain if this was a real difference in rate or simply reflected changes that had occurred in the light chains during isolation. Certainly in some cases a progressive decrease in the rate of phosphorylation by the kinase was observed after storage of whole light-chain preparations for 4–5 months. The rate of phosphorylation of the P-light chain (assayed at 7.5 mg/ml) was unchanged by the presence of the 22000- and 15000-dalton light-chain components in similar relative amounts to those present in myosin. On no occasion was phosphorylation of the 22000- and 15000-dalton light-chain components observed. In view of these facts, and as a matter of convenience, the whole light-chain fraction was used as substrate in the standard assay procedure.

Effect of Ca^{2+} and Mg^{2+} concentrations

In the absence of added Ca^{2+} from the standard incubation medium used for myosin light-chain kinase assay, the enzyme was usually fully activated by traces of endogenous Ca^{2+} in the medium. Nevertheless, 0.1 mM-$CaCl_2$ was added as a routine for assay. The enzyme was completely inhibited by the addition of 1.0 mM-EGTA to the standard incubation system. The effect of Ca^{2+} concentration on the myosin light-chain kinase activity depended

on the Mg^{2+} concentration in the assay conditions. For example, in the presence of 12.5mM-magnesium acetate the kinase was fully activated at lower Ca^{2+} concentrations than was the Mg^{2+}-stimulated ATPase of myofibrils isolated from rabbit white skeletal muscle (Fig. 4). In 6.5mM-magnesium acetate the Ca^{2+} concentrations for 50% activation of the kinase and the myofibrillar Mg^{2+}-stimulated ATPase were very similar.

If the Mg^{2+} concentration was varied in otherwise constant assay conditions, kinase activity increased until the Mg^{2+} concentration exceeded the ATP concentration and then remained constant in the presence of excess of Mg^{2+}.

Despite the presence of low concentrations of Ca^{2+} that would normally fully activate the kinase in standard assay conditions, in the absence of Mg^{2+} the kinase was virtually inactive. The Mg^{2+} requirement could not be eliminated by increasing the Ca^{2+} concentration; for example at pH7.6 with 5.5mM-ATP, 2.5mM-$MgCl_2$ and 10mM-$CaCl_2$ the kinase activity was only about 8% of that obtained in standard assay conditions, i.e. 0.1mM-$CaCl_2$ and 12.5mM-$MgCl_2$.

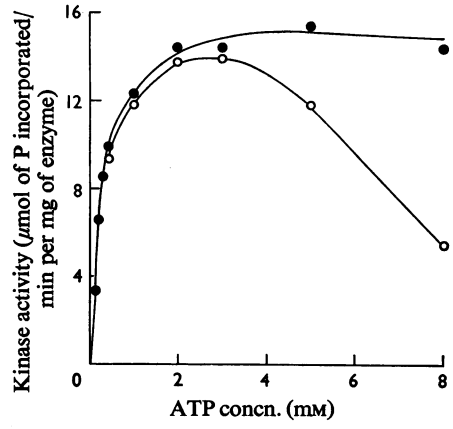

Fig. 5. *Effect of ATP and Mg^{2+} concentrations on myosin light-chain kinase activity*

Purified samples of kinase (1 μg/ml) were incubated in 50mM-Tris (adjusted to pH7.6 with 1M-HCl)/ 0.1mM-$CaCl_2$/1mM-dithiothreitol, plus whole light-chain fraction (15mg/ml), with additions indicated: ●, 12.5mM-$MgCl_2$; ○, 6.25mM-$MgCl_2$.

Nucleotide specificity

When dephosphorylated myosin with endogenous light-chain kinase activity was incubated with ATP, ITP, GTP, CTP or UTP under the standard assay conditions, phospho-(P-light chain) formation could be demonstrated by polyacrylamide-gel electrophoresis at pH8.6 in 8M-urea only with ATP as substrate. Although the method is semi-quantitative, it was estimated from the loading of the gels that phosphorylation of the P-light chain with ITP, GTP, CTP and UTP at rates less than 5% of that with ATP would be detected. Even on prolonged incubation with ITP, GTP, CTP and UTP a band corresponding to phospho-(P-light chain) could not be detected. In the presence of 12.5mM-$MgCl_2$, maximum kinase activity was reached with 2.25–3mM-ATP, at which value it remained as the ATP concentration was increased to 8mM, the highest concentration studied (Fig. 5). From the double-reciprocal-plot method (Lineweaver & Burk, 1934), somewhat variable values for the K_m with respect to ATP in the range 200–400 μM were obtained.

The results presented in Fig. 5, and those obtained in experiments in which the effect of Mg^{2+} concentration on the kinase activity in the presence of 5.5mM-ATP were studied, suggested that $MgATP^{2-}$ is the substrate and that free ATP inhibits the kinase. The latter conclusion was confirmed by the fact that the Mg^{2+} requirement for maximum kinase activity varied in a consistent way with the ATP concentration. Over the range of ATP concentration from 2.5 to 10mM, maximum myosin light-chain kinase activity was obtained when the molar ratio of Mg^{2+} to ATP was about 1.5:1. On no occasion was inhibition obtained in the presence of excess of Mg^{2+} (up to 20mM in the presence of 5.5mM-ATP).

Effect of pH

When myosin light-chain kinase was tested over the range pH5–10 a peak of activity was obtained at pH6.5, with a small shoulder in the region of pH7.0–7.5 (Fig. 6). The enzyme was reasonably stable when stored in 10% sucrose over the pH range 6.3–8.0. It was rapidly inactivated at pH values below 5.0.

Substrate specificity

Apart from its natural substrate, the P-light chains of cardiac, red skeletal and smooth muscle (Frearson & Perry, 1975; Frearson *et al.*, 1976*a*) are the only other proteins so far shown to act as effective substrates for myosin light-chain kinase from rabbit white skeletal muscle. The 'EDTA light chain' of molluscan adductor myosin (Szent-Györgi *et al.*, 1973) is not phosphorylated at a significant rate by the enzyme (Frearson *et al.*, 1976*a*). Purified preparations of myosin light-chain kinase would not catalyse the phosphorylation of the 15000- and 22000-dalton light-chain or the heavy-chain fractions of myosin from white skeletal muscle. Likewise phosphorylation could not be demonstrated with casein, calf thymus histone type 111-S, phosphorylase *b* or troponin from rabbit white skeletal muscle.

The values obtained for the K_m for the P-light chain of rabbit white skeletal muscle by using the purified

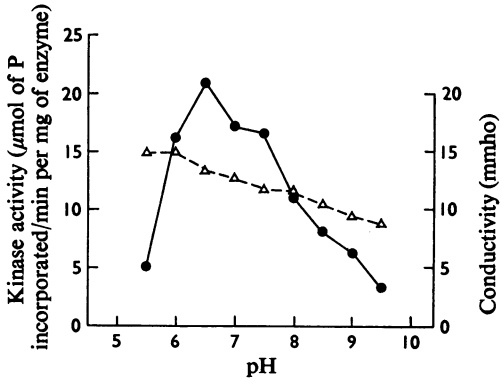

Fig. 6. *Effect of the pH on myosin light-chain kinase activity*
Purified kinase (1 μg/ml) was incubated at 25°C in 5.5 mM-ATP/12.5 mM-magnesium acetate/0.1 mM-$CaCl_2$/1 mM-dithiothreitol, plus whole light-chain fraction (15 mg/ml). The buffer (25 mM-Tris/25 mM-imidazole/25 mM-glycine) under standard assay conditions was made 4 times the final concentration, adjusted first to pH 10.0 with NaOH and then adjusted to the required pH values with HCl. ●, Kinase activity; △, conductivity.

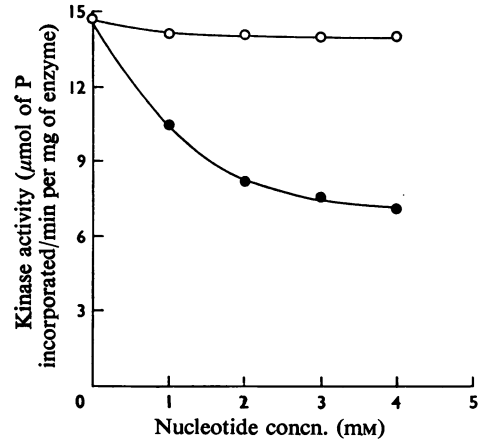

Fig. 7. *Effect of ADP* (●) *and AMP* (○) *on myosin light-chain kinase activity*
Purified samples of kinase (1 μg/ml) were incubated under the standard assay conditions.

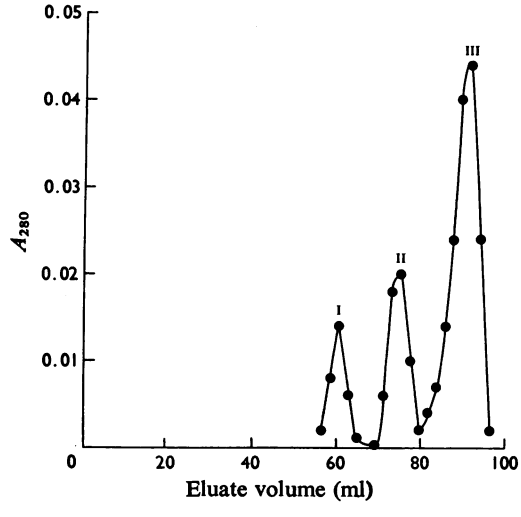

Fig. 8. *Gel filtration of pure sample of myosin light-chain kinase after storage at 2°C in phosphate buffer*
Enzyme (approx. 1.0 mg in 1 ml) that migrated on electrophoresis in sodium dodecyl sulphate as a single band corresponding to a mol.wt. of 77000 was stored for 15 days in 150 mM-phosphate buffer (pH 6.2)/1 mM-magnesium acetate / 0.25 mM-dithiothreitol. After storage it was applied to an Ultrogel AcA44 column (1.5 cm × 80 cm) equilibrated against and eluted with the same buffer. See Plate 1 (*f*)–(*h*).

enzyme from the same muscle were in the range 100–200 μM.

Modification of enzymic activity

No evidence was obtained for a system regulating activity such as is the case with the other Ca^{2+}-requiring kinase, phosphorylase kinase. Addition of 1–5 μM-3′:5′-cyclic AMP had no significant effect on the activity of the kinase at any stage of the purification. Preincubation with cyclic AMP-dependent protein kinase and cyclic AMP likewise had no effect on activity. The most effective inhibitor was EGTA, which at 1 mM completely inhibited the enzyme. Whereas AMP had no effect, ADP inhibited strongly (Fig. 7). P_i up to 0.1 M had only slight inhibitory activity (10–12%) on the crude and purified enzyme. Likewise the activity of the enzyme was not much affected by increasing the ionic strength up to 0.4 M with NaCl.

Enzymically active subfragments of myosin light-chain kinase

It was noted that on some occasions, particularly when preparations had been left at 2°C in the presence of phosphate buffer, and after chromatography on hydroxyapatite, which was also carried out in phosphate buffer, three peaks of kinase activity corresponding to proteins of mol.wts. of about 77000, 50000 and 30000 were obtained on gel filtration on Sephadex G-200 (Fig. 8, Plates 1*f*–1*h*). The protein of mol.wt. 30000 had a lower specific activity (10–15 μmol/min per mg) than the normal preparation of the purified enzyme and migrated as a rather diffuse band on electrophoresis in the presence of sodium dodecyl sulphate at pH 7.0 (Plate 1*h*). On one occasion

a purified preparation of the kinase that migrated in gels containing sodium dodecyl sulphate as a single band corresponding to a mol.wt. of 77000 when freshly prepared, migrated as three bands of apparent mol.wt. of 77000, 50000 and 30000 after storage for 15 days in 0.15M-phosphate buffer at 2°C. There were indications that degradation occurred particularly on storage in phosphate buffer (Plates 1*i* and 1*j*) and could involve complete conversion into the 30000-dalton component. Storage at −10°C arrested the degradation (Plates 1*j* and 1*k*).

Discussion

The study of the properties of the purified enzyme confirms that myosin light-chain kinase (ATP–myosin P-light-chain phosphotransferase) is not identical with the other protein kinases that have been isolated from muscle. It differs from phosphorylase kinase and 3′:5′-cyclic AMP-dependent protein kinase in molecular weight, specificity and mechanism of activation. It does have one property in common with phosphorylase kinase, however, in that low concentrations of Ca^{2+} are required for activity. When related to weight of protein, the specific activity of myosin light-chain kinase is significantly higher than those of the other two protein kinases isolated from muscle (Rubin *et al.*, 1971; Reimann *et al.*, 1971). In terms of mol of substrate turned over per mol of enzyme in unit time, however, phosphorylase kinase is more active because of its high molecular weight. Myosin light-chain kinase of muscle has a similar molecular weight to the analogous enzyme reported to be present in platelets (Daniel & Adelstein, 1976), but differences exist between the enzymes. The platelet enzyme does not require Ca^{2+} for activity, but purification of the platelet enzyme to homogeneity on a larger scale is required before detailed comparisons can be made. Daniel & Adelstein (1976) report a specific activity of about 12nmol of P incorporated/min per mg of protein for purified platelet myosin light-chain kinase, which is approx. 1/2000 of the activity of the purified muscle enzyme. It is not clear whether this represents a real difference in properties or merely a reflection of the purity of the kinase isolated from platelets. The platelet kinase does appear to differ from the muscle kinase in that light chain associated with myosin is a very much better substrate than is the isolated form (Daniel & Adelstein, 1976).

The relationship of the myosin light-chain kinase to the myosin light-chain phosphatase is of some interest in terms of both function and evolution. It was noted (Morgan *et al.*, 1976) that at the early stages of purification activity of the two enzymes fractionated together, implying that they may exist in the sarcoplasm as a complex. Their molecular weights are similar, but the activities of the two enzymes are separated at later stages of purification.

Myosin light-chain kinase possesses a number of features that require some comment. The K_m of the pure enzyme with respect to the isolated P-light chain is higher than that reported for the crude enzyme preparation (Perry *et al.*, 1976), suggesting that some modification of the enzyme may have occurred during purification. Values for the K_m obtained *in vitro* with isolated light-chain fractions may not be very relevant to the situation in muscle, where the P-light chain is localized in the head of the myosin molecule, but clearly the change in K_m with purification requires further investigation. The molecular-weight studies suggest that the enzyme exists as a monomer under ionic conditions similar to those existing in the cell. The existence of enzymically active preparations of mol.wts. of about 50000 and 30000 is suggestive of possible subunit structure. If that is the case, however, it is surprising that these lower-molecular-weight fractions could not be detected on electrophoresis in sodium dodecyl sulphate. Limited degradation of the enzyme due to slight contamination of some preparations with proteolytic enzymes, either of muscle or of bacterial origin, is a more likely explanation in view of these findings. The modification of the enzyme that may occur during preparation could explain the change in K_m values observed with purification. Whatever the explanation of the observation, the fact that enzymically active material has been obtained of apparent mol.wt. 30000 implies that the whole of the 77000-dalton molecule is not required for enzymic activity.

Although the phosphorylation–dephosphorylation system for the P-light chain of myosin is now well defined by the purification and study of the enzymes involved, the role of this process *in situ* is as yet uncertain. So far as the myosin from white skeletal muscle is concerned, the P-light chain may be fully phosphorylated in the resting stage (Perrie *et al.*, 1973), as it is in the normal heart beating with its own intrinsic rhythm and which has not been subjected to interventions to produce changes in the force developed (Frearson *et al.*, 1976*b*). From the enzymic studies *in vitro* the rise in Ca^{2+} concentration associated with contraction would favour phosphorylation. If it is assumed that the activities measured *in vitro* apply *in vivo*, the amounts of the enzymes present are such that a phosphorylation–dephosphorylation cycle could not occur during a single twitch in skeletal muscle or during a single cycle of diastole and systole in cardiac muscle. Probably in white skeletal muscle, which has a much higher kinase activity than cardiac muscle, the cycle of phosphorylation and dephosphorylation would be in the second rather than the millisecond time-scale. Study of the steady-state kinetics of the ATPase activities of phosphorylated and dephosphorylated myosin in the presence and absence of actin has not indicated any gross enzymic

differences (Morgan *et al.*, 1976). In this respect skeletal-muscle myosin appears to differ from myosin of platelets and vertebrate smooth muscle, phosphorylation of which has been reported to produce changes in the extent of activation of its ATPase by actin and in the sensitivity of the actomyosin system to Ca^{2+} (Adelstein *et al.*, 1976; Gorecka *et al.*, 1976; Sobieszek & Small, 1976).

In the perfused rabbit heart, interventions that lead to an increase in force, arising from increased intracellular Ca^{2+} concentration, also result in the dephosphorylation of the P-light chain (Frearson *et al.*, 1976*b*). After the force returns to normal the *P*-light chain is rephosphorylated (Frearson *et al.*, 1976*b*). In view of the evidence that is accumulating suggesting a role for the P-light chain in the interaction of myosin with actin, it is possible that phosphorylation may modify this interaction. At the moment the effects of phosphorylation appear to be more apparent in the slower muscles such as cardiac and smooth muscle, and the process may constitute another mechanism involving change in the sensitivity of the system regulating the interaction of actin and myosin to Ca^{2+}.

The P-light chain of myosin from vertebrate muscle may preserve some aspects of the role in Ca^{2+} regulation of the actomyosin ATPase that has been demonstrated in molluscan adductor muscles that do not contain troponin (Lehman *et al.*, 1972). It is possible that with the emergence of the troponin system in vertebrate muscle, phosphorylation of the *P*-light chain has taken on a special role in regulating the sensitivity of the actomyosin ATPase to Ca^{2+}. In cardiac muscle the phosphorylation of troponin I associated with the inotropic response leads to a decreased sensitivity of the cardiac actomyosin system to Ca^{2+} (Solaro *et al.*, 1976; Ray & England, 1976; H. A. Cole & S. V. Perry, unpublished work). By analogy, the partial dephosphorylation of the myosin that occurs after interventions leading to a rise in intracellular Ca^{2+} may also lead to a change in sensitivity of the actomyosin ATPase to Ca^{2+}. Such a hypothesis would be compatible with the preliminary report (Adelstein *et al.*, 1976) suggesting that an increase in Ca^{2+} sensitivity of the ATPase of smooth-muscle actomyosin occurs when the myosin is phosphorylated.

The work was supported in part by a grant from the Medical Research Council and during the tenure of a scholarship from the Instituto de Alta Cultura, Portugal, by E. M. V. P.

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