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Myosin Light-Chain Phosphatase

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1. A method for the isolation of a new enzyme, myosin light-chain phosphatase, from rabbit white skeletal muscle by using a Sepharose–phosphorylated myosin light-chain affinity column is described. 2. The enzyme migrated as a single component on electrophoresis in sodium dodecyl sulphate/polyacrylamide gel at pH 7.0, with apparent mol.wt. 70000. 3. The enzyme was highly specific for the phosphorylated P-light chain of myosin, had pH optima at 6.5 and 8.0 and was not inhibited by NaF. 4. A Ca^{2+}-sensitive 'ATPase' (adenosine triphosphatase) system consisting of myosin light-chain kinase, myosin light-chain phosphatase and the P-light chain is described. 5. Evidence is presented for a phosphoryl exchange between P_i, phosphorylated P-light chain and myosin light-chain phosphatase. 6. Heavy meromyosin prepared by chymotryptic digestion can be phosphorylated by myosin light-chain kinase. 7. The ATPase activities of myosin and heavy meromyosin, in the presence and absence of F-actin, were not significantly changed (±10%) by phosphorylation of the P-light chain.

It is now well established that the 18000-dalton light-chain component of myosin isolated from rabbit white skeletal muscle can be phosphorylated at a single serine residue (Perrie *et al.*, 1973) by a highly specific enzyme, myosin light-chain kinase, which has been partially purified (Pires *et al.*, 1974). This light chain has been variously designated as the DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)] light chain (Weeds, 1969; Gazith *et al.*, 1970) and the M_{l_3} light chain (Perrie & Perry, 1970).

Myosins isolated from vertebrate red skeletal and cardiac muscle (Frearson & Perry, 1975) and from vertebrate smooth muscle (Frearson *et al.*, 1976) also contain a light chain that can be phosphorylated both by a kinase endogenous to the muscle and by the partially purified kinase from rabbit white skeletal muscle. Adelstein *et al.* (1973) have demonstrated a similar phosphorylation of the 20000-dalton light chain of human platelet myosin, which leads to about a sixfold increase in the actin-stimulated hydrolysis of ATP measured in the presence of Mg^{2+} (Adelstein & Conti, 1975). Thus all the myosins so far isolated from vertebrate muscle contain a light-chain component which varies slightly in molecular weight and extractability in different muscles, but which can be distinguished from the other light-chain components by its property of acting as a specific substrate for myosin light-chain kinase. This light-chain component we have designated the 'P-light chain' (Frearson & Perry, 1975) rather than use the nomenclature referred to above which is no longer meaningful when used for myosins other than that from rabbit white skeletal muscle.

In the original studies on the P-light chain (Perrie & Perry, 1970), it was apparent that the extent to which this light chain was phosphorylated depended on the method of preparation of the myosin. Also dephosphorylation could be achieved by incubation of myosin or the isolated light-chain fraction with a low-ionic-strength extract of muscle. The present investigation of the mechanism of dephosphorylation of the P-light chain has led to the isolation from muscle of a new highly specific enzyme, which we have designated myosin light-chain phosphatase (phospho-myosin P-light-chain phosphohydrolase). The properties of the enzyme and the effect of phosphorylation on the properties of myosin are also described. Some aspects of the work have been reported (Perry *et al.*, 1975*a*,*b*).

Materials and Methods

Materials

Hydroxyapatite was prepared by the method of Bernadi (1971). DEAE-cellulose was supplied by Whatman Biochemicals, Springfield Hill, Maidstone, Kent, U.K., and Sephadex and Sepharose 4B by Pharmacia Fine Chemicals AB, Uppsala, Sweden. Bovine serum albumin, ovalbumin, transferrin, chymotrypsin, phosphorylase *b* and bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. [γ-^{32}P]ATP was supplied by The Radiochemical Centre, Amersham, Bucks., U.K.

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Preparation of muscle proteins

Dephosphorylated myosin was prepared from rabbit white skeletal muscle by the method of Pires *et al.* (1974). For light-chain preparations the myosin was precipitated four times and for crude myosin light-chain kinase and phosphatase preparations the myosin was precipitated twice. Partially phosphorylated myosin was prepared by the method of Trayer & Perry (1966). F-actin was prepared by the method of Johnson *et al.* (1967), and desensitized actomyosin as described by Syska *et al.* (1976).

Troponin was isolated from rabbit white skeletal muscle by the method of Ebashi *et al.* (1971).

Myosin light-chain fraction

Preparations of the whole dephosphorylated light-chain fraction were obtained by ethanol precipitation of rabbit myosin in 5M-guanidine hydrochloride as described by Perrie & Perry (1970). The light-chain fraction was freeze-dried from aqueous solution for storage.

A whole light-chain fraction, in which the 18000-dalton component was fully phosphorylated, was prepared as follows. Whole light-chain fraction (30.0mg/ml) was incubated with crude light-chain kinase (10–15μg/ml) in 5mM-[γ-^{32}P]ATP (4μCi/μmol), 50mM-Tris/40mM-HCl, pH7.6, 12.5mM-magnesium acetate, 0.1mM-$CaCl_2$ and 0.2mM-dithiothreitol for 30min at 25°C. The reaction was stopped by addition of 100mM-EGTA* to a final concentration of 5mM. ATP was removed by gel filtration on a Sephadex G-25 column (25cm×2.5cm) equilibrated against 50mM-Tris/40mM-HCl/10mM-β-mercaptoethanol, pH7.6. The protein peak was collected and the light-chain fraction precipitated by an equal volume of 15% (w/v) trichloroacetic acid. After being washed twice with 5% trichloroacetic acid, the phosphorylated light chains were dissolved in 25mM-Tris/20mM-HCl, pH7.6, and dialysed overnight against this buffer.

Myosin light-chain kinase. The packed suspension of twice-precipitated myosin (see above) sedimented by centrifugation in 50mM-KCl was freeze-dried and stored at −10°C. Freeze-dried myosin (4g) was dispersed in 5.0ml of 4mM-EDTA, adjusted to pH7.0 with 100mM-$NaHCO_3$, by using a hand homogenizer of the Potter type, and left for 20min at 4°C. After centrifugation for 30min at 50000*g*, the supernatant, which contained approx. 15mg of protein/ml, was removed and used as a crude extract of myosin light-chain kinase.

Preparation of phosphorylated proteins. Phosphorylase *b* kinase (1.6mg/ml), histone (8mg/ml) and casein (10mg/ml) were each incubated with 3′:5′-cyclic AMP-dependent protein kinase (0.1mg/ml), 5mM-[γ-^{32}P]ATP (4μCi/μmol), 50mM-Tris/40mM-HCl, 20mM-α-glycerophosphate, 50mM-NaF, 10mM-magnesium acetate, 0.1mM-$CaCl_2$, 0.12mM-3′:5′-cyclic AMP, pH7.2, for 30, 60 and 60min respectively.

Phosphorylase *b* (10mg/ml) and rabbit skeletal-muscle troponin (15mg/ml) were each phosphorylated by incubation with phosphorylase kinase, (160μg/ml), 50mM-Tris/20mM-HCl, 50mM-NaF, 10mM-magnesium acetate, 0.1mM-$CaCl_2$, pH8.6, for 20min and 6h respectively.

In all cases, excess of ATP was removed by gel filtration on Sephadex G-25 as described for the preparation of phosphorylated myosin light chains. The material in the protein peaks was collected, dialysed against 50mM-Tris/40mM-HCl (pH7.6)/50mM-β-mercaptoethanol and used directly as substrates for myosin light-chain phosphatase.

Preparation of Sepharose–phosphorylated light-chain affinity column

Sepharose 4B was activated as described by March *et al.* (1974) with 30mg of CNBr/g of packed Sepharose. Some 15g of activated Sepharose was added to 10ml of 50mM-Tris/40mM-HCl/10mM-magnesium acetate/15mM-dithiothreitol, pH7.6 (buffer A), containing 30–40mg of ^{32}P-labelled whole light-chain fraction of rabbit white-muscle myosin, and left for 20h at 4°C. After repeated washing by suspension in 50mM-Tris/40mM-HCl (pH7.6)/10mM-β-mercaptoethanol followed by centrifugation until significant amounts of ^{32}P were no longer present in the supernatant, the amount of phosphorylated light chain bound to the Sepharose was estimated from the amount of ^{32}P covalently bound to the Sepharose. On average approx. 1.5mg of whole light-chain fraction was bound to 1g wet wt. of packed Sepharose.

Rephosphorylation of Sepharose–myosin light chain

Sepharose (20g) to which myosin light-chain fraction had been linked was washed exhaustively with buffer A and suspended in 20ml of this buffer. Then 2ml of a crude extract of myosin light-chain kinase (350μg/ml) was added and 2ml of 50mM-ATP (sodium salt) containing 10μCi of [γ-^{32}P]ATP. The Sepharose was stirred at room temperature (21°C) for 45min and the reaction stopped by washing the Sepharose with 10vol. of 0.5M-KCl in buffer A, followed by 10vol. of buffer A. The extent of rephosphorylation was monitored by measurement of the ^{32}P covalently bound to 1g of washed Sepharose.

The rephosphorylated column was used twice, after which it was rephosphorylated as above for further use. After four rephosphorylations the column was discarded.

* Abbrevations: EGTA, ethanedioxybis(ethylamine)-tetra-acetic acid; ATPase, adenosine triphosphatase; ITPase, inosine triphosphatase.

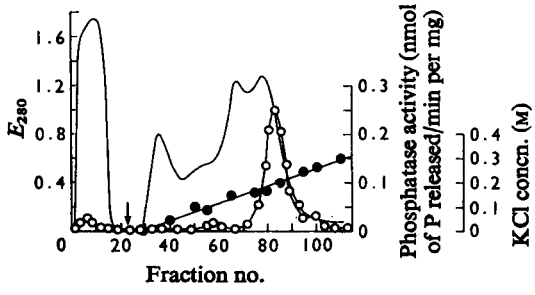


Fig. 1. *Chromatography on DEAE-cellulose of muscle protein fraction precipitated by 50%-satd. $(NH_4)_2SO_4$*

Protein fraction (60ml, 18mg/ml) in buffer A was applied to a column (6cm×12cm) of DEAE-cellulose DE-52 equilibrated against buffer A. A linear gradient was applied at the point marked by an arrow, by using 500ml mixing chambers. Fractions (8ml) were collected, and myosin light-chain phosphatase was assayed as described in the Materials and Methods section. ——, E_{280}; ○, myosin light-chain phosphatase; ●, KCl concentration.

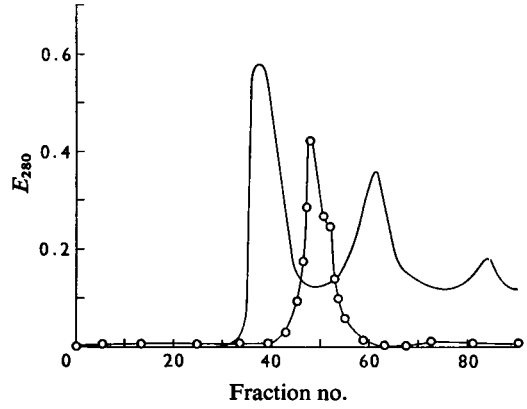


Fig. 2. *Gel filtration of fraction containing myosin light-chain phosphatase eluted from DEAE-cellulose*

A fraction (1.5ml, 13mg/ml) was dialysed against buffer A and applied to a column (95cm×1.5cm) of Sephadex G-200 equilibrated against buffer A. Fractions (3ml) were collected. ——, E_{280}; ○, myosin light-chain phosphatase activity.

Purification of myosin light-chain phosphatase

Minced muscle (400–500g) from rabbit white skeletal muscle was extracted with 2.5vol. of buffer A for 25min at 4°C. The clear supernatant obtained after centrifugation at 5000*g* for 30min was brought to 50% saturation with solid $(NH_4)_2SO_4$, the pH being adjusted to pH7.6 with aq. 1.0M-NH_3. All subsequent procedures were carried out at 4°C. After standing at 4°C for 25min, the precipitate was collected by centrifugation at 18000*g* for 30min, redissolved in 25ml of buffer A and dialysed against 2 litres of buffer A overnight. Insoluble material was removed by centrifugation at 9000*g* for 10min before application of the clear supernatant to a DEAE-cellulose column (6cm×12cm) equilibrated against buffer A.

On application of a gradient of 0–0.5M-KCl to the buffer the myosin light-chain phosphatase was eluted with a peak corresponding to 0.2M-KCl (Fig. 1). The fractions containing the phosphatase were identified in the final peak eluted from the column and were combined. The enzyme was precipitated by the addition of solid $(NH_4)_2SO_4$ to 70% saturation to the combined fractions and collected by centrifugation for 30min at 18000*g*. In larger preparations, i.e. from 1000g of minced muscle, the DEAE-cellulose-chromatography step was repeated. The protein precipitated by $(NH_4)_2SO_4$ after chromatography on DEAE-cellulose was dialysed against a solution consisting of buffer A+glycerol (1:1, v/v) and applied to a Sephadex G-200 column (95cm×1.5cm) equilibrated against buffer A (Fig. 2). The fractions containing phosphatase activity were pooled and applied to a Sepharose 4B column to which phosphorylated whole light chains had been linked (see above and the Results section). After being washed with 3vol. of buffer A the enzyme was eluted by application of 50mM-Tris/40mM-HCl/10mM-EDTA/15mM-dithiothreitol, pH7.6 (Fig. 3). The fractions representing the eluted peak of enzyme were pooled and dialysed against buffer A containing 50% glycerol. This solution of the enzyme (about 100μg/ml) kept its activity for about 5 days when stored at 4°C.

Preparation of phosphorylase kinase

Phosphorylase kinase was prepared from white skeletal muscle of the rabbit as described by Cohen (1973).

Assay of myosin light-chain phosphatase

Phosphorylated whole myosin light-chain fraction (10mg/ml) was incubated with 50mM-Tris/40mM-HCl/12.5mM-magnesium acetate/0.1mM-$CaCl_2$/0.5mM-dithiothreitol, pH7.6, at 30°C, in a total volume of 1ml. The reaction was usually stopped by addition of 1ml of 15% (w/v) trichloroacetic acid at timed intervals up to 30min. With phosphorylated histone as substrate the reaction was stopped by the addition of 1ml of 80mM-silicotungstic acid in 40mM-H_2SO_4. The protein precipitate was removed by centrifugation at 4°C for 30min at 4000*g*. ^{32}P was determined in the supernatant by Čerenkov counting

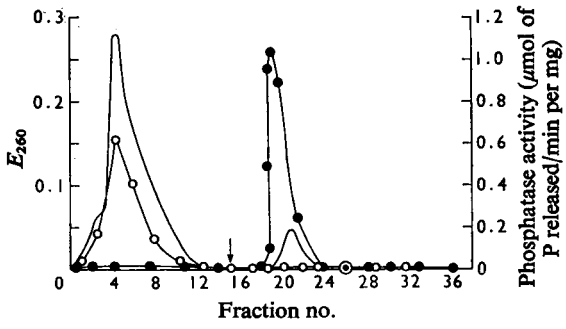


Fig. 3. *Chromatography of myosin light-chain phosphatase preparation on a Sepharose–phosphorylated light-chain column*

The fraction (30ml, 0.3mg/ml) with phosphatase activity obtained by gel filtration (see Fig. 2) was applied to a Sepharose–phosphorylated light-chain column (12cm×2cm) previously equilibrated with buffer A. The column was washed with buffer A until the E_{280} of the eluate was zero. A step of 50mM-Tris/40mM-HCl/10mM-EDTA/15mM-dithiothreitol, pH7.6, was applied at the point marked with an arrow. ——, E_{280}; ○, phosphorylase *a* phosphatase; ●, myosin light-chain phosphatase.

(Perry & Cole, 1973). ^{35}S was determined by scintillation counting by using 0.5ml samples added to 9.5ml of scintillation fluid consisting of 2 parts of toluene, containing 2,5-diphenyloxazole (4g/litre) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.12g/litre), and 1 part of Triton X-100. All determinations were corrected for controls, which were subjected to the same procedure without the addition of phosphatase.

Assay of phosphorylase a phosphatase

Samples (0.1ml) of fractions eluted from the columns were incubated with 12.5mM-magnesium acetate, 0.1mM-$CaCl_2$, 2mM-dithiothreitol, 50mM-Tris/40mM-HCl, pH7.6, and ^{32}P-labelled phosphorylase *a* (3mg/ml); the total volume was 1ml, and the temperature was 30°C. The reaction was stopped by addition of 1ml of 15% trichloroacetic acid after 15min, and ^{32}P was measured in the supernatant as for myosin light-chain phosphatase.

Assays of nucleoside triphosphatases

Enzyme activities were determined under the following conditions: (1) Ca^{2+}-stimulated ATPase: 5mM-$CaCl_2$, 0.25M-KCl, 50mM-Tris/40mM-HCl, pH7.6, 2.5mM-ATP; (2) Mg^{2+}-stimulated ATPase: 25mM-Tris/20mM-HCl, pH7.6, 2.5mM-$MgCl_2$, 2.5mM-ATP; (3) K^+/EDTA-stimulated ATPase: 0.6M-KCl, 5mM-EDTA, 50mM-Tris/40mM-HCl, pH 7.6, 2.5mM-ATP. ITPase assays were carried out with 2.5mM-ITP, 5mM-$CaCl_2$ or 2.5mM-$MgCl_2$; conditions were otherwise as indicated in (1) and (2).

In all cases myosin concentrations of 0.3–0.6mg/ml or equivalent amounts of heavy meromyosin were used. Stock F-actin solutions (10–15mg/ml) in 5mM-Tris/4mM-HCl, pH7.6, were added to produce a ratio of 1 part by weight of actin to 4 of myosin. The total volume was 2ml. Incubations were carried out for 5min at 25°C and stopped by the addition of 1ml of 15% (w/v) trichloroacetic acid.

Actomyosin centrifugation experiments

Myosin (20mg/ml) was fully phosphorylated with [γ-^{32}P]ATP by using the endogenous myosin light-chain kinase activity by incubation for 15min at 25°C under standard conditions (12.5mM-magnesium acetate, 0.1mM-$CaCl_2$, 50mM-Tris/40mM-HCl, pH 7.6, 0.2mM-dithiothreitol, 5.0mM-ATP). The reaction was stopped by the addition of 10vol. of cold water. The precipitated myosin was sedimented by centrifugation for 10min at 4000*g* and dissolved by the addition of solid KCl to a final concentration of 0.6M. Precipitation was repeated and the fully phosphorylated myosin was then added to an equal amount of dephosphorylated myosin dissolved in 0.6M-KCl, adjusted to pH7.6 with $NaHCO_3$ so that the final protein solution (10mg/ml) contained myosin of which 50% of the molecules were fully phosphorylated.

Actomyosins in which the myosin/actin ratio varied from 10:1 (w/w) to 1:1 (w/w) were prepared by adding 0.1ml of F-actin solution in 5mM-Tris/4mM-HCl, pH7.6, of various concentrations to 1ml of 50% (w/v) phosphorylated myosin. The KCl concentration was brought to 0.28M by the addition of 1.3ml of water, and the actomyosin formed was left on ice for 5min before being centrifuged for 20min at 30000*g*. The actomyosin formed was sedimented, and the excess of myosin or actin remained in the supernatant (shown by control experiments).

Samples of the supernatant and pellet were removed for determination of ^{32}P by the Čerenkov method (Perry & Cole, 1973). The composition of the samples was also examined by polyacrylamide-gel electrophoresis (in 6M-urea, pH8.6).

Calibration of Sephadex column for molecular-weight determination

A Sephadex G-200 column (95cm×1.5cm) equilibrated against buffer A was calibrated by using 3.0mg of transferrin, 5.0mg of bovine serum albumin and 3.0mg of ovalbumin. Void volume V_0 (Andrews, 1965) was determined with Blue Dextran; flow rate was about 8ml/h.

Protein determinations

These were carried out by the method of Lowry *et al.* (1951) or for dilute enzyme solutions by the method of Mejbaum-Katzenellenbogen & Dobryszycka (1959). Both methods were standardized against bovine serum albumin.

Electrophoresis

Electrophoresis was carried out as described by Cummins & Perry (1973) on 10% polyacrylamide gels in 82.5mM-Tris/400mM-boric acid, pH7.0, containing 0.1% sodium dodecyl sulphate. Before electrophoresis, protein standards ovalbumin, transferrin, catalase, phosphorylase, myosin heavy chains and bovine serum albumin were equilibrated with the sodium dodecyl sulphate buffer by boiling the samples for10min.

Polyacrylamide-gel electrophoresis was also carried out in 6M-urea/25mM-Tris/122mM-glycine, pH8.6, as described by Perrie & Perry (1970).

Results

Purification of myosin light-chain phosphatase

When protein solutions obtained by extracting freeze-dried myosin with 4mM-EDTA, pH7.0, were chromatographed on DEAE-cellulose, two peaks of myosin light-chain phosphatase activity were observed (Perry *et al.*, 1975*a*). Myosin light-chain kinase activity was associated with each of the two peaks of phosphatase activity under these conditions. Two similar peaks containing kinase and phosphatase activity were also observed when partially phosphorylated myosin was chromatographed on DEAE-cellulose or on hydroxyapatite under the conditions described in Fig. 4. This procedure did not consistently separate the phosphorylated and non-phosphorylated forms of myosin, but in all cases peaks of myosin light-chain phosphatase activity were present in the ascending and descending regions of the myosin peak.

Although myosin light-chain phosphatase activity was usually present in preparations of myosin that had been precipitated two to four times, the bulk of the enzyme activity was present in the supernatant obtained after centrifuging a homogenate of mixed skeletal muscle of the rabbit in 2.5vol. of 50mM-Tris/40mM-HCl (pH7.6)/10mM-magnesium acetate/15mM-dithiothreitol. This extract was used, therefore, for the preparations of the phosphatase (see the Materials and Methods section).

The purification achieved at the different stages of the preparation is summarized in Table 1. Figs. 1 and 2 illustrate the distribution of enzymic activity in the eluate from the DEAE-cellulose and Sephadex G-200 columns respectively. Although about 800-fold purification was achieved up to the gel-filtration stage, the fraction containing myosin light-chain phosphatase also possessed phosphorylase *a* phosphatase activity.

The final purification stage on the Sepharose 4B–phosphorylated light-chain affinity column completely removed phosphorylase *a* phosphatase

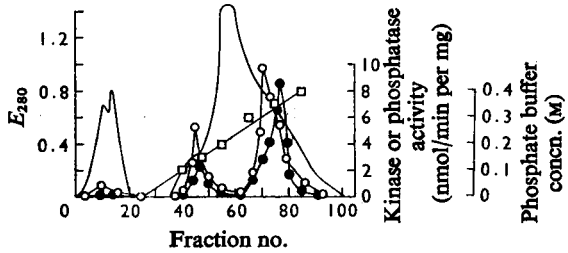


Fig. 4. *Chromatography of myosin from rabbit white muscle on hydroxyapatite*

Partially phosphorylated myosin (100mg) dissolved in 10ml of 20mM-sodium pyrophosphate, pH7.6, was applied to a hydroxyapatite column (12cm×3cm) equilibrated against the same solution. A gradient was applied to a concentration of 0.5M-sodium phosphate buffer, pH7.6, by using 2×100ml mixing chambers. Fractions (5ml) were collected. Enzyme activities were measured as indicated in the Materials and Methods section. ——, E_{280}; ○, phosphatase; ●, kinase; □, sodium phosphate buffer concentration.

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

Initial extract was made from 300g of mixed rabbit skeletal muscle as described in the Materials and Methods section.

	Volume (ml)	Activity (nmol/min per mg)	Protein (mg/ml)	Purification (×)
Initial extract	850	0.11	35.0	
50%-satd. $(NH_4)_2SO_4$ precipitate	100	0.253	50.0	2.3
Fraction eluted from DEAE-cellulose	67	2.64	4.7	24
70%-satd. $(NH_4)_2SO_4$ precipitate	6	6.93	15.0	63
Fraction eluted from Sephadex G-200	20	91.3	0.3	830
Fraction eluted from Sepharose 4B column	10	1200	0.02	10800

activity (Fig. 3). The latter enzyme was not held on the column, whereas myosin light-chain phosphatase was eluted by the application of 50mM-Tris/40mM-HCl/10mM-EDTA/15mM-dithiothreitol (pH7.6) as a single peak with specific activities in the range of 1–2 μmol/min per mg when measured under standard conditions, i.e. about 120–130-fold purification. On polyacrylamide-gel electrophoresis at pH7.0 in sodium dodecyl sulphate, 30 μg of the enzyme preparation migrated as a single component; in 6M-urea, pH8.6, it migrated as one main band and a slower minor band representing about 5% of the total protein (Plate 1). Although providing an effective purification step which yielded virtually pure enzyme, the yields from the affinity columns were low, about 300 μg of enzyme from a 20g column. The columns were dephosphorylated rather rapidly during use and were normally used twice. For re-use they were rephosphorylated with myosin light-chain kinase (see the Materials and Methods section).

Properties of myosin light-chain phosphatase

When assayed under standard assay conditions (see the Materials and Methods section) with enzyme concentrations in the range 5–10 μg/ml, rates were independent of substrate concentrations of whole light-chain fraction $\geq$10mg/ml. Assuming that the whole light-chain fraction of myosin from rabbit white skeletal muscle contained 50% of its weight of P-light chain of mol.wt. 18500, a value of 48 μM was obtained for the Michaelis constant, determined by the method of Cornish-Bowden & Eisenthal (1974).

Optimum activities were obtained at pH6.5 and pH8.0, with a minimum at pH7.0 (Fig. 5). After dialysis against 50mM-Tris/25mM-HCl, pH7.6, containing 15mM-dithiothreitol the enzyme was inactive when tested in the absence of added bivalent cations. Activity was restored by Mg^{2+}, Mn^{2+} or Co^{2+}, and, although it was the highest in the presence of Mg^{2+}, it was not restored to the value obtained before dialysis (Fig. 6). Addition of up to 50mM-NaF to the standard assay conditions in the presence of Mg^{2+} produced less than 5% inhibition. This suggests that the enzyme does not require Mg^{2+} ions for activity. Nevertheless, in the absence of Mg^{2+}, dilute solutions of the enzyme lost activity rapidly, and it appears that the cation stabilized the enzyme. The activity in the presence of Mg^{2+} was not significantly affected by 5mM-EGTA, implying that traces of Ca^{2+} were not essential for activity under these conditions.

The enzyme was highly specific for the P-light chain. No other substrate was hydrolysed at a significant rate, i.e. more than 5% of that obtained with the P-light chain of myosin from rabbit white muscle. Substrates tested were: *p*-nitrophenyl phosphate, α- and β-glycerophosphate; the phosphorylated form of glycogen synthetase and phosphorylase kinase phosphorylated on the α and β subunits; histones F1 and F2b, casein and phosphorylase kinase, all phosphorylated with bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase; phosphorylase *a*, troponin complex, troponin I, all phosphorylated with phosphorylase kinase. Although myosin light-chain kinase transfers the thiophosphate group from AMP-PP(S) to the P-light chain (Perry *et al.*, 1975*a*), the thiophosphorylated light chain produced was a very poor substrate for myosin light-chain phosphatase (Fig. 7).

The purified enzyme was inhibited slightly by P_i. In contrast, the myosin light-chain phosphatase activity of extracts of whole muscle obtained by extraction in 5 vol. of buffer A followed by centrifugation was markedly inhibited by P_i. With 15mM-potassium phosphate buffer, pH7.0, 80% inhibition of myosin light-chain phosphatase activity of whole-muscle extracts was obtained (Fig. 8).

P_i exchange

When the purified myosin light-chain phosphatase was incubated with $^{32}P_i$, ^{32}P was incorporated into

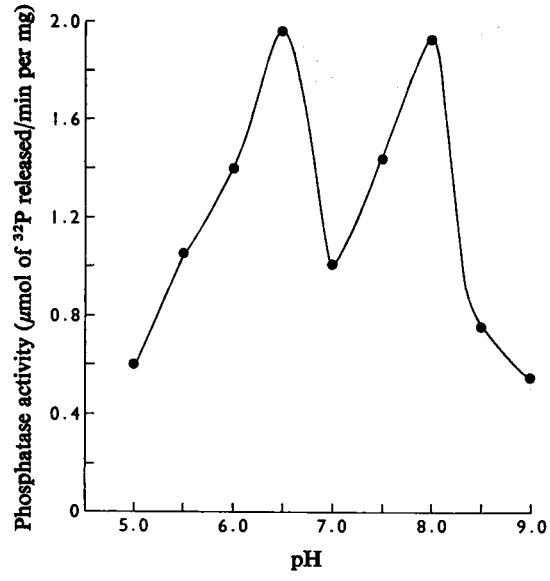
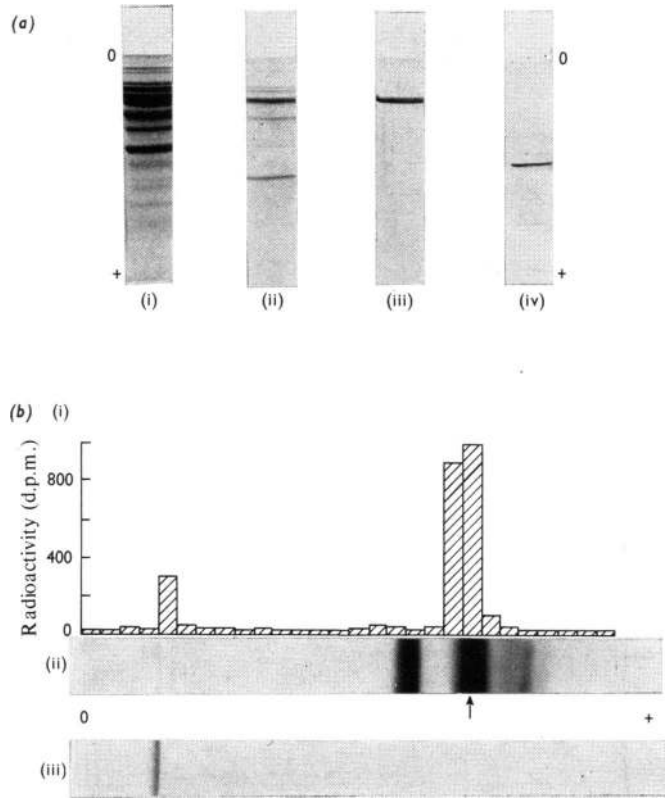


Fig. 5. *Effect of pH on the activity of myosin light-chain phosphatase*

Assay conditions were 100mM-Tris/citrate buffer (stock buffer at pH values used made by addition of 1M-citric acid to 1M-Tris), 10mM-magnesium acetate, 0.1mM-$CaCl_2$, 1mM-dithiothreitol, phosphorylated whole light chain (10mg/ml), affinity-column-purified enzyme; conditions were otherwise as described in the Materials and Methods section. Points represent the average of two experiments carried out on different enzyme preparations.



EXPLANATION OF PLATE 1

(a) Electrophoresis of myosin light-chain phosphatase preparations and (b) phosphorylation of myosin light-chain phosphatase by incubation with ^{32}P-labelled light-chain fraction

In (*a*): (i) 20μg of active fraction eluted from DEAE-cellulose (Fig. 1), sodium dodecyl sulphate, pH7.0; (ii) 50μg of active fraction eluted from Sephadex G-200 (Fig. 2), sodium dodecyl sulphate, pH7.0; (iii) 50μg of enzyme eluted from the affinity column (Fig. 3), sodium dodecyl sulphate, pH7.0; (iv) 25μg of enzyme eluted from the affinity column (Fig. 3), 6M-urea, pH8.6. In (*b*) the incubation conditions were 12.5mM-magnesium acetate, 0.1mM-$CaCl_2$, 50mM-Tris/40mM-HCl, pH7.6, 0.2mM-dithiothreitol, 10mg of ^{32}P-labelled whole light-chain fraction, 50μg of myosin light-chain phosphatase; total volume was 1 ml. The reaction stopped after 30min by the addition of 1 ml of sodium dodecyl sulphate/borate buffer system, pH7.0, used for electrophoresis; 100μl was applied to polyacrylamide gel for electrophoresis. (i) Distribution of radioactivity along the polyacrylamide gel illustrated in (ii). Gel sliced in 5mm strips which were dissolved in 2.0ml of 80% (v/v) H_2O_2 for ^{32}P determination; (ii) 100μl of whole incubation mixture/sodium dodecyl sulphate, pH7.0; phosphorylated P-light chain is indicated by an arrow. Additional bands other than phosphatase and myosin light chains were due to components in the crude myosin light-chain kinase preparation used for phosphorylation of the light-chain fraction; (iii) 35μg of myosin light-chain phosphatase/sodium dodecyl sulphate, pH7.0.

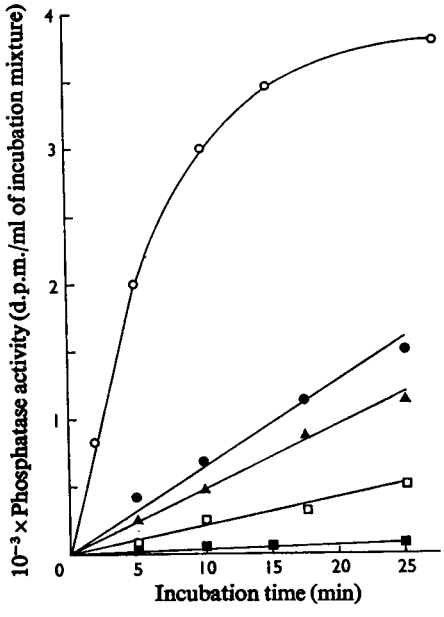


Fig. 6. *Effect of metal ions on the activity of myosin light-chain phosphatase*

Solution of enzyme (100 μg/ml) in 25 mM-Tris/20 mM-HCl (pH 7.6)/10 mM-magnesium acetate/15 mM-dithiothreitol was dialysed overnight against 25 mM-Tris/20 mM-HCl (pH 7.6)/15 mM-dithiothreitol. Incubation conditions: light chains (5 mg/ml), 50 mM-Tris/40 mM-HCl, pH 7.6, 0.2 mM-dithiothreitol, myosin light-chain phosphatase (10 μg/ml). ○, Undialysed enzyme, 5 mM-magnesium acetate; ●, dialysed enzyme, 5 mM-magnesium acetate; ▲, dialysed enzyme, 5 mM-$MnCl_2$; □, dialysed enzyme, 5 mM-$CoCl_2$; ■, dialysed enzyme, no addition.

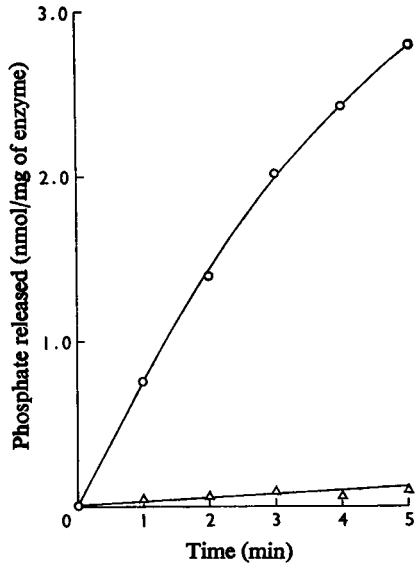


Fig. 7. *Comparison of rates of dephosphorylation of phosphorylated and thiophosphorylated myosin P-light chain*

Whole myosin light-chain fraction (3 mg/ml) from rabbit white skeletal muscle was incubated with [γ-^{32}P]ATP (5 mCi/mmol) or ^{35}S-labelled AMP-PP(S) (0.05 mCi/mmol) under the conditions described for phosphorylation of actomyosin in the Materials and Methods section. Both samples were checked by electrophoresis in 6 M-urea, pH 8.6, for complete phosphorylation. Phosphorylated and thiophosphorylated light chains (2.5 mg/ml) were incubated with 12.5 mM-magnesium acetate, 0.1 mM-$CaCl_2$, 50 mM-Tris/40 mM-HCl, pH 7.6, 0.2 mM-dithiothreitol and crude myosin light-chain phosphatase (0.5 mg/ml, 50% $(NH_4)_2SO_4$ precipitate) in a total volume of 1 ml. The reaction was stopped by addition of 1.0 ml of 15% (w/v) trichloroacetic acid, and ^{32}P and ^{35}S were measured in the clear supernatant after centrifugation. ○, Phosphorylated light chains; △, thiophosphorylated light chains.

the enzyme. The extent of incorporation increased with time, reaching a maximum corresponding to about 0.8 mol of P/70000 g after about 60 min incubation at 25°C (Fig. 9). The ^{32}P could not be removed from the enzyme by repeated washing with 7.5% (w/v) trichloroacetic acid containing 5 mM-potassium phosphate buffer, pH 7.0. By analogy with the behaviour of the alkaline phosphatase of *Escherichia coli* (Engstrom, 1959), this effect was considered to represent the phosphorylation of a serine (or threonine) residue of the enzyme.

On polyacrylamide-gel electrophoresis of the enzyme in sodium dodecyl sulphate, pH 7.0, after incubation with P_i, the single band obtained was labelled with ^{32}P. Both of the bands obtained on electrophoresis in 6 M-urea at pH 8.6 were labelled. The minor band of the two, which represented <5% of the total protein, was apparently more strongly labelled. Owing to the small amounts of material, the distribution of radioactivity between the two bands observed could not be satisfactorily quantified.

The property of incorporating covalently bound ^{32}P on incubation with P_i presented a convenient method of identifying myosin light-chain phosphatase in crude extracts. Radioautography of electrophoretograms of whole-muscle extracts revealed several protein bands labelled with ^{32}P, in addition to myosin light-chain phosphatase. These presumably represented other enzymes present in whole-muscle extracts that could exchange with P_i. When ^{32}P-labelled light chains were incubated with the enzyme under standard conditions for myosin light-chain phosphatase assay, the enzyme became radioactive, suggesting that a phosphorylated intermediate was formed (Plate 1*b*).

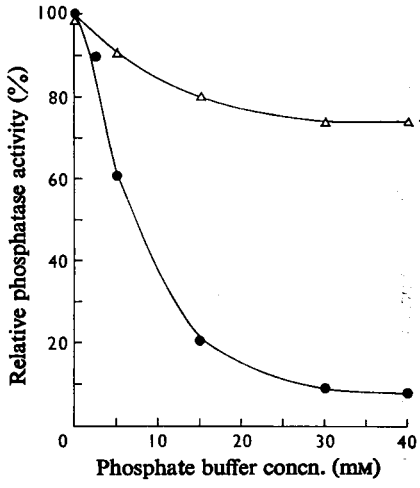


Fig. 8. *Effect of P_i on the myosin light-chain phosphatase activity of extracts of whole muscle and the purified enzyme*

Assays were carried out in 50mM-Tris/40mM-HCl, pH7.6, 12.5mM-magnesium acetate, 0.1mM-$CaCl_2$, 0.2mM-dithiothreitol and phosphorylated whole light-chain fraction (5mg/ml), myosin light chain phosphatase (10μg/ml) or extract of whole muscle prepared as described in the text (1mg/ml). Potassium phosphate buffer, pH7.0, was added to give the phosphate concentrations indicated. The total volume was 1.0ml. △, Purified myosin light-chain kinase; ●, whole muscle extract.

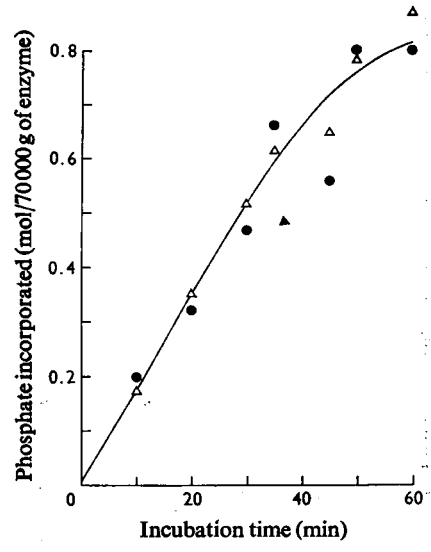


Fig. 9. *Uptake of phosphate by myosin light-chain phosphatase*

The incubation mixture contained myosin light-chain phosphatase (20μg), 50mM-Tris/40mM-HCl, pH7.6, 12.5mM-magnesium acetate, 0.1mM-$CaCl_2$, 0.2mM-dithiothreitol, 2mM-sodium phosphate buffer, pH7.6 (5μCi of ^{32}P/mmol), in a total volume of 1ml. The incubation was at 25°C for the times indicated. Trichloroacetic acid (1.0ml, 15%) was added, followed by 3mg of serum albumin in 1ml of water as carrier. The protein pellet was washed three times with 5% trichloroacetic acid containing 0.1M-sodium phosphate buffer, pH7.6. The protein was dissolved in 1.0ml of 0.1M-NaOH and made up to a total volume of 6.0ml with water for ^{32}P determination. The two symbols indicate experiments with different enzyme preparations.

Combined action of myosin light-chain kinase and phosphatase

As has been mentioned above, when EDTA extracts of freeze-dried myosin or whole myosin were chromatographed on DEAE-cellulose, two peaks of myosin light-chain phosphatase were obtained (Fig. 4). Myosin kinase activity was also associated with each peak, and if myosin light chains were present, 'ATPase' activity could be demonstrated when assays were carried out in the presence of 12.5mM-$MgCl_2$/0.1mM-$CaCl_2$/5mM-ATP at pH7.6 (Perry *et al.*, 1975*a*). This 'ATPase' activity was presumed to be due to the combined action of the two enzyme systems, with the P-light chain acting as a coenzyme.

$$\text{P-light chain} + \text{ATP} \underset{}{\overset{\text{kinase}}{\rightleftharpoons}} \text{phosphorylated P-light chain} + \text{ADP}$$

$$\text{Phosphorylated P-light chain} + \text{water} \overset{\text{phosphatase}}{\rightleftharpoons} \text{P-light chain} + P_i$$

In some cases when myosin, or EDTA extracts of it, were chromatographed and the kinase and phosphatase peaks did not separate, peaks of ATPase activity could be detected, particularly at the trailing edge of the main peak (cf. Fig. 4, Perry *et al.*, 1975*a*).

Control experiments with purified myosin light-chain kinase and phosphatase indicated that the mechanism proposed above was responsible for the 'ATPase' activity. The experiment illustrated in Table 2 demonstrates that 'ATPase' activity could be obtained only in the presence of both enzymes and the P-light chain. As the myosin light-chain kinase requires Ca^{2+} for activity the 'ATPase' activity is Ca^{2+}-sensitive.

Effect of phosphorylation on the biological activity of myosin

For study of the effect of phosphorylation on the enzymic properties of myosin, thrice-precipitated dephosphorylated myosin (15–20mg/ml) was incubated with 12.5mM-magnesium acetate, 0.1mM-$CaCl_2$, 50mM-Tris/40mM-HCl, pH7.6, 2mM-dithiothreitol and 5mM-ATP, and the reaction was stopped after 10–15min by the addition of 0.1M-EGTA to bring the final concentration of EGTA to 5mM.

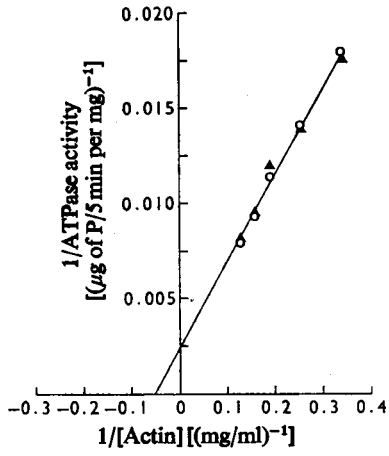


Fig. 10. *Effect of F-actin on the Mg^{2+}-stimulated ATPase of phosphorylated and dephosphorylated heavy meromyosin*

Heavy meromyosin was prepared by chymotryptic digestion as described in the Materials and Methods section. Heavy meromyosin (300μg/ml) was incubated with F-actin (0–10mg/ml) in a total volume of 1ml containing 25mM-Tris/20mM-HCl, pH7.6, 2.5mM-magnesium acetate, 2.5mM-ATP and 1mM-dithiothreitol. Results were plotted as described by Eisenberg & Moos (1968). ▲, Dephosphorylated heavy meromyosin; ○, phosphorylated heavy meromyosin.

Under these conditions the P-light chain was completely converted into its phosphorylated form by endogenous myosin light-chain kinase. Control unphosphorylated myosin samples were treated in exactly the same way, except that the EGTA concentration was adjusted to 5mM to inhibit the myosin light-chain kinase at the beginning of the incubation. When controls were carried out in this way there was no change in ATPase activity due to incubation alone, but if incubations were carried out in the absence of ATP before assay, a significant loss in ATPase activity occurred. The state of phosphorylation of the myosin in control and experimental incubations was checked by polyacrylamide-gel electrophoresis in 6M-urea, pH8.6.

There were no significant differences between the ATPase activities of myosin in the phosphorylated and unphosphorylated forms in the presence of Ca^{2+}, Mg^{2+} and K^+/EDTA (see the Materials and Methods section). These results were obtained when phosphorylation and ATPase assays were carried out in the presence or absence of 10mM-2-mercaptoethanol. Similar results were obtained with heavy meromyosin prepared by chymotryptic digestion (Leadbeater & Perry, 1963). Heavy meromyosin prepared by the procedure of Szent-Gyorgyi (1953) by using a ratio of trypsin/myosin of 1:1000 was not phosphorylated either when incubated alone under the conditions described above or when crude myosin light-chain kinase (10mg/ml) was added. Tryptic digestion appeared to have destroyed the myosin light-chain kinase associated with the myosin and modified the P-light chain so that it could no longer be phosphorylated by the added kinase. In an earlier investigation it was shown that when heavy meromyosin is prepared by chymotryptic digestion, the enhancement of the Mg^{2+}-stimulated ATPase obtained in the presence of actin is much greater than when trypsin is used. It was therefore concluded that the proteolysis with chymotrypsin was less extensive (Leadbeater & Perry, 1963). This conclusion was supported by the fact that when heavy meromyosin was prepared by the Leadbeater & Perry (1963) procedure, the P-light chain was preserved and, although endogenous myosin light-chain kinase activity was destroyed, the P-light chain was phosphorylated in the presence of added myosin light-chain kinase. Weeds & Taylor (1975) have also reported the preservation of the P-light chain in heavy meromyosin prepared by chymotrypic digestion.

Table 2. *Ca^{2+}-sensitive ATPase obtained by combined action of myosin light-chain kinase and phosphatase in the presence of the P-light chain*

Incubations were carried out in 1ml at pH7.6 in 12.5mM-magnesium acetate, 0.1mM-$CaCl_2$, 50mM-Tris/40mM-HCl, pH7.6, 2.5mM-ATP, 0.2mM-dithiothreitol; dephosphorylated whole light chains (5mg), kinase (60μg) and phosphatase preparation (250μg) were added as indicated.

Additions	P_i released (μg/min per ml)
Light chains	2.5
Light chains + kinase	1.5
Light chains + phosphatase	1.7
Kinase	0.8
Phosphatase	0.7
Kinase + phosphatase	1.0
Light chains + kinase + phosphatase	54.0
Light chains + kinase + phosphatase + 5mM-EGTA	8.0

The hydrolysis of ITP in the presence of Ca^{2+} (1.5 ± 0.3 μmol of P/min per mg of myosin) and Mg^{2+} (0.02 ± 0.01 μmol of P/min per mg of myosin) were likewise not significantly affected by phosphorylation of the myosin.

In normal preparations of desensitized actomyosin, the P-light chain is completely dephosphorylated. Phosphorylation of these preparations did not affect the Ca^{2+}, Mg^{2+} or K^+/EDTA-stimulated ATPase. When incubated with 60μg of troponin I from rabbit white skeletal muscle in a total volume of 2ml under the conditions for Mg^{2+}-stimulated ATPase (see the Materials and Methods section), dephosphorylated desensitized actomyosin was inhibited $20 \pm 5\%$ in the

absence and 80±2% in the presence of 45μg of tropomyosin. The extent of inhibition was not significantly changed by phosphorylation of the desensitized actomyosin. This was carried out as described for myosin, except that 0.1M-KCl was added to the incubation medium to decrease the Mg^{2+}-stimulated ATPase activity, which rapidly lowers the ATP concentration under the standard conditions used for phosphorylation by myosin light-chain kinase.

The interaction of myosin or heavy meromyosin with actin was not significantly modified by phosphorylation so far as could be judged from the effect of actin on the Mg^{2+}-stimulated ATPase. Double-reciprocal plots over the the range 10–100mM-KCl, carried out as described by Eisenberg & Moos (1968), indicated no significant differences in apparent dissociation constant or $V_{max.}$ whether myosin or chymotrypsin-prepared heavy meromyosin was used (Fig. 10).

The ATPase activities of acto-heavy meromyosin in the presence of Ca^{2+} and K^+/EDTA were also not significantly affected by phosphorylation.

The interaction of actin with myosin studied by centrifuging down actomyosin complexes from solutions containing various proportions of actin and myosin likewise gave no evidence that there was any selective binding of phosphorylated or dephosphorylated forms of myosin to actin.

Discussion

Myosin light-chain phosphatase clearly appears to be a unique enzyme highly specific for the P-light chain of myosin. Although the studies described have been directed to the enzyme present in 'fast' white muscle of the rabbit, a similar enzyme, which has not yet been purified, is present in other muscles (Frearson *et al.*, 1976).

When purified, the enzyme can exist as a monomeric form of mol.wt. about 70000. With less pure preparations, however, two peaks of phosphatase activity were obtained on gel filtration and ion-exchange chromatography. The observation that myosin light-chain kinase activity was associated with both peaks of phosphatase activity suggests that the two enzymes may exist in some form of complex, either with themselves or possibly with other components of sarcoplasm, to form a higher-molecular-weight aggregate.

Although the system has not yet been investigated in detail, the rapid incorporation of phosphate on incubation of the enzyme with P suggests, by analogy with the alkaline phosphatase of *E. coli* (Engstrom, 1959), that the enzyme can catalyse the transfer of the phosphoryl group between water and a specific residue, presumably serine, at the active site. This phosphorylation is probably a reflexion of the involvement of the phosphorylated enzyme in the catalytic mechanism of myosin light-chain phosphatase. It is, to our knowledge, the first example of a protein phosphatase in which an equilibrium has been demonstrated between the phosphorylated form and P_i.

The existence of highly specific enzymes for the phosphorylation and dephosphorylation of one serine residue on the P-light chain of all vertebrate muscle myosins naturally raises the question of the function of the process. In all other cases of well-characterized enzymes that undergo phosphorylation and dephosphorylation, e.g. phosphorylase (Fischer & Krebs, 1955), glycogen synthetase (Villar-Palasi & Larner, 1961; Wieland & Von Jagow-Westerman, 1969) and pyruvate dehydrogenase (Linn *et al.*, 1969), the catalytic activity is markedly affected by the state of phosphorylation of the enzyme. Despite an exhaustive series of experiments, we have been unable to demonstrate significant differences in enzymic activity of phosphorylated and dephosphorylated forms of myosin and heavy meromyosin, incubated either with substrate alone or in the presence of actin. In this respect our results differ from those reported by Adelstein & Conti (1975) with platelet myosin. Likewise, studies to compare the binding of actin with that of myosin and heavy meromyosin also failed to demonstrate any marked change due to phosphorylation of the P-light chain. In all the measurements carried out so far the results obtained with the phosphorylated and non-phosphorylated forms of myosin did not differ by more than ±10%. It is possible that phosphorylation produces a minor change in properties, which is not detected by the level of precision at which the studies reported were carried out.

There is evidence that the P-light chain of vertebrate myosin may be involved in the interaction with actin. Such a role is supported by the studies of Margossian *et al.* (1975) and by the fact that actin activates the Mg^{2+}-stimulated ATPase of chymotrypsin-prepared heavy meromyosin much more effectively than that of trypsin-prepared heavy meromyosin (Leadbeater & Perry, 1963), in which the P-light chain is modified. If the P-light chain has such a role, the addition of two negative charges or a specific site on each light chain would be expected to have an effect on the interaction. Such an effect, however, is not revealed by the relatively crude techniques used in the study.

Although the physiological function of the phosphorylation of myosin from skeletal muscle is as yet unsolved, it is noteworthy that the combined action of the myosin light-chain kinase and phosphatase in the presence of P-light chain, either free or associated with myosin, will bring about the rapid hydrolysis of ATP. As the kinase requires Ca^{2+} this 'ATPase' activity is EGTA-sensitive, and when adequate

amounts of the enzymes are present the rate of hydrolysis is high. In view of the fact that both enzymes contaminate normal myosin preparations, it is necessary to demonstrate that such a system is not responsible for the apparent Ca^{2+} regulation obtained in some actomyosin systems in the absence of troponin. It is probable that this system accounted for the previously unexplained observation reported by Perry (1960) that frequently two peaks of specific ATPase activity are obtained when myosin is chromatographed on DEAE-cellulose.

We are grateful to Dr. P. Cohen for gifts of phosphorylated glycogen synthetase, phosphorylase kinase and histones F1 and F2b and to Dr. F. Eckstein for gifts of ^{35}S-labelled AMP-PP(S). Our thanks are also due to Mr. A. J. Harborne who carried out preliminary experiments as a final-year undergraduate research project on the feasibility of using the Sepharose–phosphorylated light-chain system for enzyme purification. This work was supported in part by a grant from the Medical Research Council.

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