MYOSIN LIGHT-CHAIN KINASE, A NEW ENZYME FROM STRIATED MUSCLE

E. PIRES, S. V. PERRY and M. A. W. THOMAS*
Department of Biochemistry, University of Birmingham, Birmingham B15 2TT, U.K.

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1. Introduction

It has been shown that white skeletal muscle of the rabbit contains an enzyme that transfers the γ phosphate group from ATP to each of the two 18 000 dalton light chains of myosin isolated from this tissue [1–3]. Although the function of this phosphorylation is as yet unknown there is good evidence that myosin is present in the phosphorylated form in resting muscle and that an enzyme is present in this tissue which can bring about its dephosphorylation [3, 4].

In our preliminary experiments we reported that the dephosphorylated form of the 18 000 dalton light chain, M_3 (nomenclature of Perrie and Perry [4]) either when separated from myosin or associated with the intact molecule, was converted to the phosphorylated form, M_2, by incubation with preparations of phosphorylase kinase such as the '40-precipitate fraction' of Krebs et al. [5] and occasionally with protein kinase. During the course of this study however, a number of observations suggested that the enzyme involved in the phosphorylation of the myosin light chain was not identical with either of these well-characterised enzymes. For example, the amino acid sequence of the phosphopeptide of 13 residues isolated from chymotryptic digests of M_2 [3] is very different from that of the phosphopeptide isolated from phosphorylase [6].

It was also noted that a crude phosphorylase kinase preparation that catalysed the transfer of the γ phosphate group of ATP to the 18 000 dalton light chain component of rabbit white skeletal muscle was also able to phosphorylate the 19 000 dalton light chain component of a red skeletal muscle, such as the crureus, but was apparently less effective in phosphorylating the 19 000 dalton light chain of cardiac myosin. In other respects the cardiac myosin light chain component that was phosphorylated appeared similar to the 19 000 dalton light chain component of crureus muscle [7]. Crude whole sarcoplasm from rabbit white skeletal muscle was, however, effective in transferring the γ phosphate of ATP to the 19 000 dalton light chain of myosin of rabbit cardiac muscle [7].

As an extension of these findings we wish to report the presence of a kinase present in sarcoplasm, but also associated with three times precipitated myosin, that is not identical with either phosphorylase kinase or protein kinase. This enzyme, which we have provisionally named myosin light chain kinase i.e. ATP: myosin light chain phosphotransferase, catalyses the phosphorylation of the light chain of molecular weight 18 000–19 000 that is present in the myosin isolated from skeletal and cardiac muscles.

2. Methods and materials

2.1. *Protein preparations*

All preparations were carried out at 4°C unless otherwise indicated. Myosin that contained about 30% of the M_2 component was obtained from rabbit white skeletal muscle (i.e. dorsi and leg) as described by

* Present address: Department of Biochemistry, University of Sydney, Sydney 2006, N.S.W. Australia.

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Perrie et al. [3]. Myosin in which all the 18 000 dalton light chain component was in the dephosphorylated form (Ml_3-myosin) was prepared by extracting minced L. dorsi and leg muscles of the rabbit with 2 vol of 0.6 M KCl, 15 mM 2-mercaptoethanol, adjusted to pH 7.0 with saturated sodium bicarbonate solution and then proceeding in the standard manner [8]. This procedure gave less pure preparations of myosin from red skeletal and cardiac muscles but the preparations were adequate for identification of the light chain components. The complete light chain fraction of myosin was isolated from three-times precipitated Ml_3 myson by the method of Perrie et al. [3].

The method of Ebashi et al. [9] was used for the preparation of troponin from white skeletal muscle of the rabbit. It was fractionated into its components as described by Perry and Cole [10].

Phosphorylase kinase was prepared from rabbit white skeletal muscle by the method of Cohen [11] with the modification that the initial extract was centrifuged at 2000 *g* rather than 6000 *g*. A crude preparation used in some studies was obtained by the addition of ammonium sulphate to 35% saturation to the '30 supernatant fraction' of Krebs et al. [5]. The precipitate obtained in this way was also subjected to further fractionation by the procedure of Cohen [11].

The myosin suspension in 0.05 M KCl after two preparations was freeze-dried. 8 g were extracted with 100 ml of 4 mM EDTA, 10 mM 2-mercaptoethanol, pH 7.0, in a pestle-type homogeniser. The extract after centrifugation for 45 min at 30 000 *g* was dialysed overnight against 4 mM EDTA, 0.25 sucrose, 1 mM DTT and then centrifuged for 30 min at 30 000 *g* to give about 70 ml of clear supernatant of E_{280} = 0.5–1.0. This extract was used as a crude preparation of myosin light chain kinase and was purified further as described in the text.

2.2. *Assays and determinations*

Phosphorylase kinase and protein kinase assays were carried out as described by Cohen [11] and Reimann et al. [12] respectively. Protein was determined by the method of Lowry et al. [13].

Protein precipitates and acrylamide gels were prepared for ^{32}P determination by the Cerenkov method as described previously [10,14]. [γ-^{32}P]ATP was supplied by the Radiochemical Centre, Amersham, Bucks, U.K.

3. Results and discussion

Crude phosphorylase kinase preparations were further fractionated on Sepharose 4B as described by Cohen [11] and the ability of the peaks obtained to catalyse the phosphorylation of the Ml_3 component of myosin was determined. This was carried out by incubating the protein in the peaks (fig. 1) with a sample of Ml_3-myosin which had low endogenous myosin light-chain kinase activity and observing the formation of Ml_2 by polyacrylamide electrophoresis in 6 M urea, pH 8.6 [3]. When incubation was carried out in 25 mM Tris, 25 mM sodium glycerophosphate (pH 7.8), 1.25 mM theophyline, 12.5 mM magnesium acetate, 0.1 mM $CaCl_2$ and 5 mM ATP, approximately 200 μg of protein from peak III converted all the Ml_3 component in 5 mg of Ml_3-myosin from rabbit skeletal muscle to Ml_2 in 5 min at 30°C. Protein from peaks I and II did not effect significant amounts of transformation under identical conditions. Peak II consisted essentially of phosphorylase kinase and on electrophoresis on 5% polyacrylamide gel in 85 mM Tris–400 mM boric acid (pH 7.0), 0.05% sodium dodecyl sulphate it migrated as four bands corresponding to the α, α', β and γ components of phosphorylase kinase described by Cohen [11]. Under identical conditions of electrophoresis the protein in Peak III mi-

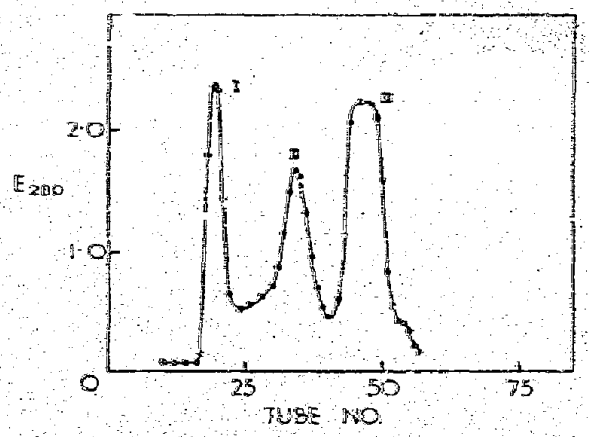

Fig. 1. Gel filtration of phosphorylase kinase. The enzyme prepared from 600 g rabbit muscle by the method of Cohen [11] was dialysed against 50 mM sodium glycerophosphate buffer (pH 7.0), 2 mM EDTA, 1 mM DTT and applied to a column of Sepharose 4B (1.5 × 90 cm) equilibrated against the same buffer.

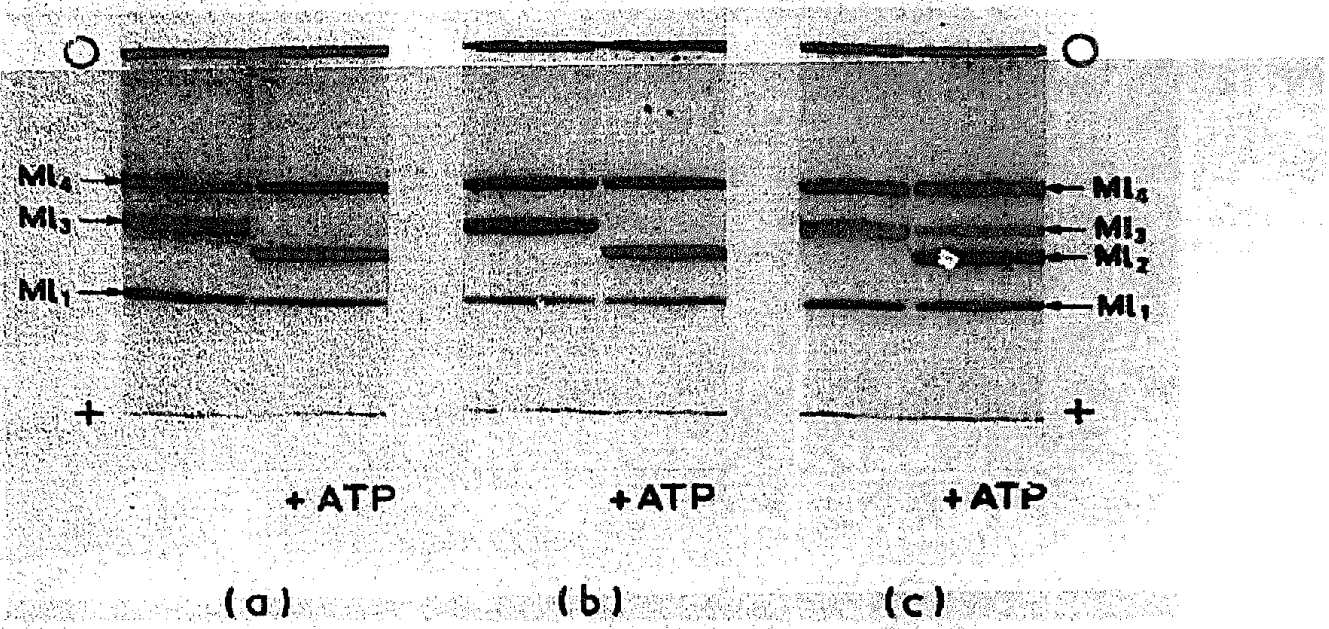

Fig. 2. Myosin light chain kinase activity of reprecipitated myosin. 4 mg myosin in 0.5 M KCl incubated in 25 mM Tris– 25 mM glycerophosphate (pH 7.6), 12.5 mM magnesium acetate, 1 mM DTT, 0.1 mM $CaCl_2$, 5 mM ATP. Controls illustrated on left of each pair of gels, incubated without ATP. Total vol. 1 ml, 10 min incubation at 25°C. Reaction stopped with solid urea (0.5 g/ml) and 70 μl applied to 8% polyacrylamide gel, 6 M urea, 20 mM Tris–80 mM glycine (pH 8.6) O, origin. (a) 200 μg 1 × ppted myosin. (b) 200 μg 2 × ppted myosin. (c) 200 μg 3 × ppted myosin.

grated principally as one band corresponding to a molecular weight of about 92 000. As this fraction possessed phosphorylase activity similar to that reported for the pure enzyme [15] it was considered to consist mainly of this protein.

Some purification of myosin light chain kinase was achieved by subjecting peak III material to further gel filtration. Difficulties in purification were experienced, however, as the myosin light-chain kinase was only a minor component and a more satisfactory preparation for further purification was obtained by direct extraction of freeze-dried myosin.

Early in these investigations it was noted that when freshly prepared three-times precipitated myosin, which contained little Ml_2, was incubated with 5 mM ATP, 0.1 mM $CaCl_2$, 12.5 mM magnesium acetate, 25 mM Tris 25 mM sodium glycerophosphate (pH 7.8) all the Ml_3 was converted into Ml_2, after 15–30 min at 25°C. On storage at 0°C, however, the myosin light chain kinase activity of myosin preparations often decreased. Comparison of the rates of phosphorylation on incubation of myosin with ATP under the conditions described in fig. 2, indicated that the light chain kinase activity decreased with successive reprecipitation during the preparation. Therefore, as a compromise between yield and purity, twice precipitated myosin was taken as starting material for preparation of the kinase. Although the enzyme could be extracted from myosin gels by treatment with 4 mM EDTA more concentrated extracts were obtained from freeze-dried myosin.

On subsequent dialysis of the crude enzyme extract against 4 mM EDTA 10 mM 2-mercaptoethanol, 0.25 M sucrose, pH 7.0, about one third of the protein was precipitated. This was discarded as it had low myosin light-chain kinase activity and consisted mainly of myosin. The supernatant was brought to 50% saturation with solid ammonium sulphate at pH 7.0 and the precipitate which contained the enzyme, dialysed against 50 mM sodium glycerophosphate buffer (pH 7.0), 2 mM EDTA, 1 mM DTT. On application to a Sepharose 6B column equilibrated against the same buffer the eluate profile was as illustrated in fig. 3. Myosin light chain kinase activity assayed by

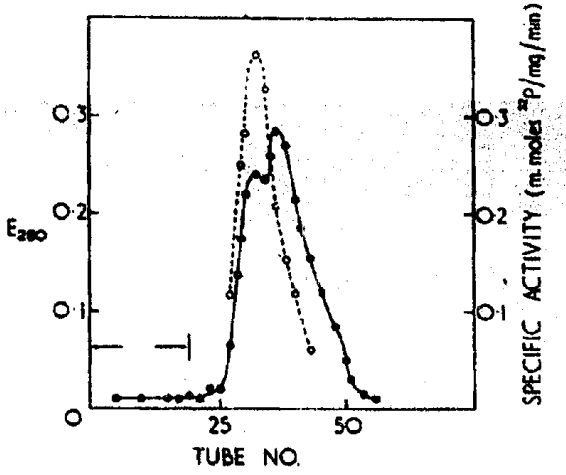

Fig. 3. Gel filtration of myosin light-chain kinase preparation. Enzyme prepared from 17 g of freeze-dried myosin as described in text, dissolved in 50 mM sodium glycerophosphate buffer (pH 7.0), 2 mM EDTA, 1 mM DTT was applied to a Sepharose 6B column (1.5 × 90 cm) equilibrated against the same buffer. Activity assayed in 25 mM Tris–25 mM sodium glycerophosphate (pH 7.6), 12.5 mM $MgCl_2$, 1 mM DTT, 0.1 mM $CaCl_2$, 10 mg/ml whole light chain fraction of myosin, 20–50 μg/ml enzyme, 5 mM [γ-^{32}P]ATP (1 μCi/μmole). Incubated 5 min at 25°C; 0.1 ml used for determination of ^{32}P. Void vol. indicated by arrows. ○, E_{280}; ●, specific activity.

the rate of incorporation of covalently bound ^{32}P into the protein of the whole light chain fraction isolated from white skeletal muscle myosin was associated with the first peak to be eluted, Fraction A (see fig. 3). At pH 7.6 1 ml of this fraction of $E_{280} = 1$ (i.e. approximately 1 mg) transferred 0.3–0.8 μmoles of ^{32}P per min to the light chain fraction of myosin when assayed as described in fig. 3. The level of activity is comparable with that of bovine cardiac protein kinase with protamine as substrate at pH 7.0 [16].

When judged qualitatively by conversion of M_3 into M_2 observed by polyacrylamide gel electrophoresis of whole myosin in 6 M urea at pH 8.6, the enzyme possessed maximum activity in the range of pH 7–8. It was inactive in the absence of $CaCl_2$, i.e. with 1 mM EGTA added to the incubation medium described in fig. 3. Addition of 3′–5′ cyclic AMP produced no significant effect on the activity either in the absence or presence of $CaCl_2$.

On electrophoresis in 7% polyacrylamide, 15% glycerol, 6.2 mM Tris 20 mM glycine buffer (pH 8.6) the preparation migrated after 4 hr at 15 mV/cm as one main diffuse band that moved only a short distance from the origin, and a minor well-defined slightly faster band. In 0.1% sodium dodecyl sulphate, 100 mM sodium phosphate buffer (pH 7.0) the protein present in Fraction A migrated as a main component of apparent molecular weight 140 000 and two faster minor components. Although it has not yet been possible to identify the component or components with which the enzymic activity is associated, the molecular weights of the components present suggested that the enzyme was not identical with phosphorylase kinase [11] nor with protein kinase [12]. This conclusion was confirmed by the inability of myosin light-chain kinase to catalyse the transfer of ^{32}P to phosphorylase *b*, troponin and casein when incubated at pH 7.6 with 5mM [γ-^{32}P]ATP. The preparation catalysed the transfer of ^{32}P from [γ-^{32}P]ATP to the whole light chain fraction of myosin from rabbit cardiac muscle (N. Frearson and S. V. Perry, unpublished observations).

We conclude that myosin light-chain kinase differs in several properties from phosphorylase kinase and protein kinase and represents an enzyme not previously reported in the literature. The extreme sensitivity of its activity to Ca^{2+} is similar to that of the troponin–tropomyosin regulatory system and implies that it will be fully active during that stage of the contractile–relaxation cycle when the myofibrillar adenosine triphosphatase activity is high. Its function cannot as yet be stated but this correlation strongly suggests some role in the chemical events occurring during muscle activity as does the fact that light chains that can be phosphorylated are found in all types of myosin from striated muscle [3] and in myosin from blood platelets [17]. The close association of the enzyme with myosin purified by the standard procedures may also be of significance. As myosin light chain kinase was previously unrecognized it was probably present as a contaminant in practically all of the myosin preparations which have to date been used for kinetic studies of ATPase activity. The significance of the presence of an active system such as this, also using ATP as substrate, for the interpretation of the results of such investigations, particularly in those studies on the transient state kinetics, requires no emphasis.

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