Phosphorylation of the Light-Chain Components of Myosin from Cardiac and Red Skeletal Muscles

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(Received 25 April 1975)

1. The light-chain components of myosin from cardiac muscle (19000 and 27000 daltons) and of rabbit soleus and crureus muscles (19000, 27000 and 29000 daltons) were characterized. 2. The 19000-dalton components in cardiac- and red-skeletal-muscle myosins were spontaneously modified to a component of slightly higher net negative charge. 3. The 19000-dalton component in cardiac and red skeletal muscles and their modified forms were phosphorylated by myosin light-chain kinase. 4. Evidence was obtained for the presence of myosin light-chain kinase in cardiac and red skeletal muscles. 5. Myosin light-chain kinase catalysed the phosphorylation of the whole light-chain fraction from white and red skeletal muscle at similar rates. The light-chain fraction of cardiac-muscle myosin was phosphorylated at a significantly lower rate. 6. The light-chain components of cardiac-muscle myosin and their phosphorylated forms were separated by ion-exchange chromatography and their amino acid compositions determined.

We have reported (Perrie *et al.*, 1972*a,b*; 1973*a,b*) that the light-chain component of mol.wt. 18500 present in myosin from white skeletal muscle, sometimes called the 'DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] light chain', can undergo phosphorylation and dephosphorylation. A highly specific enzyme, myosin light-chain kinase, catalysing the transfer of the γ phosphate of ATP to a single serine residue in the 18500-dalton component has been partially purified (Pires *et al.*, 1974) and a protein phosphatase which removes this phosphate group is also present in muscle (Perry *et al.*, 1975). These facts, coupled with the observation that the activity of myosin light-chain kinase is controlled by the Ca^{2+} concentration (Pires *et al.*, 1974), imply that phosphorylation of the 18500-dalton light chain is of metabolic significance and may be involved in the regulation of the biological activity of myosin. Preliminary studies reported elsewhere (Perrie *et al.*, 1973*b*) indicated that a light-chain component of 18000–19000 daltons that could be phosphorylated was also present in myosin in other types of striated mammalian muscle. The present paper extends and confirms this preliminary report and deals with the characterization and properties of the light-chain components of the myosins of cardiac and red skeletal muscle that are phosphorylated by myosin light-chain kinase.

Methods

Preparation of myosin

Myosin from rabbit skeletal muscle used as a source of myosin light-chain kinase preparations was prepared by extraction of minced back and leg muscles

of the rabbit with 0.6M-KCl as described by Pires *et al.* (1974). After two reprecipitations by dilution to 0.05M-KCl with distilled water the myosin precipitate was suspended in 0.05M-KCl, centrifuged for 30 min at 30000*g* and the pellet obtained was freeze-dried and stored at -10°C . Myosin light-chain kinase was extracted from freeze-dried myosin as described by Pires *et al.*, (1974).

Cardiac-muscle myosin was prepared from the rabbit and other species by two methods. For myosin that was not phosphorylated 15–50 g of minced cardiac muscle was extracted with 2 vol. of 0.6M-KCl–15 mM-2-mercaptoethanol (pH 7.0) as described by Pires *et al.* (1974). After two precipitations by dilution with distilled water actin contamination was removed by the method of Awan *et al.* (1972) and the myosin reprecipitated a further four times and stored as a gel in 0.05M-KCl at 0°C . Partially phosphorylated cardiac-muscle myosin preparations were obtained by a similar procedure which differed only in that the initial extraction was carried out with 3 vol. of 0.5M-KCl – 0.10M- KH_2PO_4 – 0.05M- K_2HPO_4 (pH 6.5) – 10 mM-sodium pyrophosphate–1 mM- MgCl_2 –10 mM-2-mercaptoethanol (Perrie & Perry, 1970). It was necessary to precipitate the myosin obtained by these two methods six times in all to remove most of the tropomyosin and other proteins that migrated with electrophoretic mobilities similar to those of the light chains.

Preparation of light chains from myosin

The whole light-chain fraction of myosin was prepared from rabbit skeletal muscle by the guanidine hydrochloride–ethanol fractionation procedure

described by Perrie & Perry (1970). For the large-scale preparations, e.g. 100–200 mg, myosin was extracted by this procedure from the muscle residue obtained from the first stage of troponin preparations from skeletal muscle (Ebashi *et al.*, 1971) and cardiac muscle (Tsukui & Ebashi, 1973).

The unphosphorylated 18 500-dalton component of rabbit skeletal myosin M₁ [nomenclature of Perrie & Perry (1970)] was separated from the whole light-chain fraction by chromatography on DEAE-cellulose as described by Perrie *et al.* (1973a).

Electrophoresis

Polyacrylamide-gel electrophoresis was performed on vertical slabs (Perrie *et al.*, 1973a) by using 20 mM-Tris–122 mM-glycine buffer, pH 8.6, containing 8 M-urea (Perrie & Perry, 1970) or 85 mM-Tris–400 mM-boric acid buffer, pH 7.0, containing 0.1% sodium dodecyl sulphate.

For studies on the mobility of the light-chain components in different concentrations of polyacrylamide gel containing 8 M-urea and 20 mM-Tris–122 mM-glycine, pH 8.6, samples were applied to separate gels in glass tubes (8 cm × 0.5 cm). All gels used in these studies were made from the same stock acrylamide solution; since only relative, not absolute, mobilities were measured this was considered to be the only precaution necessary (cf. Rodbard & Chrambach, 1971). Densitometric scanning was carried out with a Unicam SP.500 spectrophotometer equipped with Gilford linear transporter, absorbance indicator and recorder (Gilford Instrument Laboratories, Oberlin, Ohio, U.S.A.).

Incubations with myosin light-chain kinase

Unless otherwise specified the substrate, either myosin (20 mg/ml) or the whole light-chain fraction (10 mg/ml) or individual light chains (5 mg/ml) was incubated with the enzyme (20–50 µg/ml) in 25 mM-Tris–20 mM-HCl (pH 7.6)–12.5 mM-MgCl₂–1 mM-dithiothreitol–0.1 mM-CaCl₂ at 25°C for 5 min. The reaction was initiated by addition of ATP (5 mM) or [γ -³²P]ATP (The Radiochemical Centre, Amersham, Bucks., U.K.; 5 mM; approx. 1 µCi/µmol) and terminated by addition of either solid urea (approx. 0.5 g/ml) if the sample was to be subjected to gel electrophoresis or 5% (w/v) trichloroacetic acid if its radioactivity was to be measured.

Determination of ³¹P and ³²P

Protein precipitates and acrylamide gels were prepared for ³²P determination by the Cerenkov method as described previously (Perry & Cole, 1973, 1974). Total phosphate was determined by the methods described by Perry & Cole (1974).

Results

Light chains of cardiac-muscle myosin

All samples of cardiac-muscle myosin obtained from rabbit, pig, cow and rat exhibited two light chains on polyacrylamide-gel electrophoresis in sodium dodecyl sulphate. The mobilities of the two light chains in all species studied corresponded to molecular weights of about 19 000 and 27 000 when compared with proteins of known molecular weight. Although the electrophoretic pattern of cardiac-muscle myosin in sodium dodecyl sulphate was unaffected by the history of the myosin preparation this was not the case when electrophoresis was carried out in 8 M-urea at pH 8.6. Under these conditions the light chains of myosin freshly prepared in the unphosphorylated form from fresh and deep-frozen hearts (see the Methods section) migrated as a single band of mol.wt. 27 000 and a faster closely spaced doublet corresponding to the 19 000-dalton component (Plate 1a, gels i and ii). On storage at 0°C of a suspension of myosin in 0.05 M-KCl, pH 7.6, the number of bands corresponding to the 19 000-dalton component increased and similar changes occurred more slowly in the 27 000-dalton component. Comparable results were obtained with unphosphorylated myosin from the hearts of the pig and the cow. Rat cardiac-muscle myosin was different from other cardiac-muscle myosins studied in that when freshly prepared the 19 000-dalton component migrated as a single band (Plate 1a, gel iv). On storage of rat cardiac-muscle myosin at 4°C, this band was slowly converted into a doublet after 3–4 days.

It was concluded that in unphosphorylated myosin the 19 000-dalton doublet was probably formed by a charge modification of the original form for the following reasons.

1. The proportion of the forms of the 19 000-dalton component appeared when estimated visually to change relative to each other, but the total amount of 19 000-dalton component was constant relative to the 27 000-dalton component.

2. Graphical analysis of the electrophoretic mobilities of the two bands of the doublet (Hedrick & Smith, 1968) gave two parallel lines (Fig. 1). Although this method is not sensitive to small differences in molecular weight it does indicate that the two species were of similar molecular weight but of different charge. Calculations (see the Appendix) were consistent with a valence difference of approx. 1.

3. On electrophoresis in sodium dodecyl sulphate, samples of the 19 000-dalton component that were resolved as a doublet in 8 M-urea, pH 8.6, migrated as a single band.

4. The faster of the two forms was not a phosphorylated form of the slower because its formation occurred spontaneously in the absence of ATP, and direct analysis (Table 1) showed that significant

EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis of the light-chain components of myosin from cardiac and red skeletal muscles

Electrophoresis was carried out in 20mM-Tris-122mM-glycine (pH 8.6)-8M-urea. Protein loadings are approximate. O, origin. (a) Electrophoresis of whole myosin: (i) 125 μ g of unphosphorylated myosin extracted from rabbit heart with 0.6M-KCl; (ii) 125 μ g of partially phosphorylated rabbit heart myosin; (iii) 75 μ g of unphosphorylated ox heart myosin; (iv) 100 μ g of unphosphorylated rat heart myosin; (v) 100 μ g of unphosphorylated myosin from a mixture of rabbit soleus and crureus muscles; (vi) 125 μ g of cardiac-muscle myosin contaminated with tropomyosin (indicated with an arrow) and partially phosphorylated by incubation with myosin light-chain kinase under standard conditions (see the Methods section); (vii) radioautograph of the gel illustrated in (vi). The bands correspond to the phosphorylated 19000-dalton light chain (P-19K) and the phosphorylated modified 19000-dalton light chain (P-19K'). The latter is not readily seen in (vi), cf. also (ii). (b) Electrophoresis of light chains isolated from cardiac muscle as illustrated in Fig. 4: (i) 20 μ g of mixture of unmodified and modified 19000-dalton component (19K+19K'); (ii) 20 μ g of 19000-dalton component (19K); (iii) 20 μ g of phosphorylated 19000-dalton component (P-19K); (iv) 10 μ g of phosphorylated modified 19000-dalton component (P-19K'); (v) 8 μ g of 27000-dalton component.

Fig. 1. Electrophoretic mobilities of the different forms of the 19000-dalton light-chain component of rabbit cardiac-muscle myosin in gels of different acrylamide concentrations

Mobilities are expressed relative to that of Bromophenol Blue. Electrophoresis was carried out in 8M-urea-20mM-Tris-122mM-glycine, pH8.6. See under 'Phosphorylation of myosin' for further explanation of the different forms of the 19000-dalton light-chain component. ○, Unphosphorylated unmodified form (19K); ●, unphosphorylated modified form (19K'); △, phosphorylated unmodified form (P-19K); ▲, phosphorylated modified form (P-19K').

Table 1. Total P content of light-chain components separated from partially phosphorylated rabbit cardiac-muscle myosin

Light-chain components were separated as indicated in Fig. 4 and analysed for total P content as described in the Methods section.

Mol.wt. of light-chain components	No. of measurements	P content (mol/mol)
27000	2	0.20
19000 (19K)	5	0.10
(19K')	2	0.25
(P-19K)	3	0.96
(P-19K')	2	0.95

amounts of P were not present in either of these two 19000-dalton components.

From densitometric scanning of the bands obtained on electrophoresis it was estimated that approximately equimolar amounts of the 27000-dalton and the 19000-dalton components were present per mol of rabbit cardiac-muscle myosin (Table 2).

Light chains of myosin from red skeletal muscle

When examined by gel electrophoresis in sodium dodecyl sulphate in 85 mM-Tris-400 mM-borate buffer, pH7.0, the light chains of myosin isolated from both soleus and crureus muscles of the rabbit migrated as three bands corresponding to mol.wt. 29000, 27000 and 19000. The two faster bands possessed very similar mobilities to the two light chains present in cardiac-muscle myosin. Although in some preparations the 19000-dalton component of myosin from red skeletal muscle migrated as a single band in 8M-urea at pH8.6, it usually appeared as a doublet (Plate 1a, gel v).

Densitometry of the polyacrylamide gels obtained on electrophoresis in urea and in sodium dodecyl sulphate suggested that slightly more than 1 mol of the 27000-dalton component and slightly less than 1 mol of the 29000-dalton component were present for each 2 mol of 19000-dalton light chain (Table 2).

Phosphorylation of myosin

In addition to the spontaneous modifications described above, which were probably non-enzymic, other differences in the appearance of the 19000-dalton component on electrophoresis in 8M-urea, pH8.6, which depended on the conditions of myosin extraction from cardiac or red skeletal muscle, were observed. These changes in the light-chain band pattern of cardiac or red skeletal muscle were confined to the 19000-dalton component and were analogous to those reported for myosin from white

Table 2. Amounts of light-chain components present in myosins from rabbit cardiac and red skeletal muscles

Values are the average of six determinations in each case.

Muscle type	Component (mol.wt.)	Electrophoresis in urea at pH8.6		Electrophoresis in sodium dodecyl sulphate, pH7.0	
		Percentage of total light-chain fraction*	Content (mol/mol of myosin)†	Percentage of total light-chain fraction*	Content (mol/mol of myosin)†
Cardiac	19000	45.1	2.15	40.1	1.95
	27000	54.9	1.84	60.0	2.05
Red skeletal (Soleus and crureus combined)	19000	39.6	1.95	41.5	2.04
	27000	37.5	1.30	29.5	1.02
	29000	22.9	0.74	29.0	0.94

* Amounts determined by densitometry, assuming that all components stained similarly with the dye.

† Assuming myosin contains 4 mol of light chain/mol.

skeletal muscle (Perrie & Perry, 1970; Perrie *et al.*, 1973a).

To prepare myosin free of actin and tropomyosin, particularly from frozen hearts, it is necessary to include pyrophosphate or ATP in the initial extraction buffer. When prepared in this way (see the Methods section), the 19000-dalton component invariably migrated as four bands on electrophoresis in 8M-urea, pH8.6. If extracted with salt solutions in the absence of phosphate or actomyosin-dissociating reagents, the myosin was less pure and contaminating proteins were present in addition to the doublet described above. By analogy with myosin from white skeletal muscle of the rabbit, in which the 18500-dalton light chain and a modified form of it, the so-called 'satellite band' (Perrie *et al.*, 1973a), were both phosphorylated by myosin light-chain kinase, it was concluded that the two additional bands with mol.wt. 19000 were probably phosphorylated forms of the two species of 19000-dalton component of cardiac-

muscle myosin represented by the doublet seen in preparations of unphosphorylated myosin.

This conclusion was confirmed by incubating unphosphorylated rabbit cardiac-muscle myosin with myosin light-chain kinase isolated from rabbit skeletal muscle. Under these conditions the original doublet was converted into a four-banded pattern by the appearance of a new doublet of slightly faster mobility than the original, the amounts of which increased with the time of incubation. The mobilities of the newly formed doublet bands corresponded to those of the two additional bands observed in cardiac-muscle myosin prepared in pyrophosphate solutions. When a similar experiment was carried out with [γ - 32 P]ATP, significant radioactivity was detected by radioautography of the gel, or by sectioning the gel and counting the radioactivity that was present only in the region of the two new bands (Plate 1a, gels vi, vii.)

Measurements of the mobilities of each of the 19000-dalton bands were consistent with four polypeptide chains of approximately equal size, each differing from the next by a valence difference of 1 (see Fig. 1 and the Appendix). Thus we conclude that the four bands are (in order starting with that migrating nearest to the cathode): the original 19000-dalton component (19K), its 'satellite' (19K'), the phosphorylated component (P-19K) and the phosphorylated 'satellite' form (P-19K'). The band migrating most rapidly to the anode, P-19K', often faint after staining, was not readily recorded by photography (cf. Plate 1a, gels ii, vi). It could, however, be readily detected by eye and particularly by radioautography after phosphorylation with [γ - 32 P]ATP. The relative intensities of the bands illustrated in Plate 1(a) (gel vii) do not accurately represent the relative amounts of the two forms of phosphorylated light chain present as the radioautograph was intentionally over-exposed to intensify the band corresponding to form P-19K'. The four-band pattern obtained with partially phosphorylated cardiac-muscle myosin from all species studied is illustrated diagrammatically in Fig. 2.

When rat cardiac-muscle myosin exhibiting a single 19000-dalton component (i.e. 19K form) was incubated with rabbit myosin light-chain kinase a new band appeared, the electrophoretic mobility of which corresponded to the P-19K form. If, however, the rat cardiac-muscle myosin was aged at 4°C so that the 19000-dalton component spontaneously changed into a doublet (i.e. 19K and 19K' forms), two additional bands were produced on incubation with ATP and myosin light-chain kinase [i.e. P-19K and P-19K' forms (Fig. 2)]. Observations similar to those described for cardiac-muscle myosin were obtained with myosin isolated from either the soleus or crureus muscles of the rabbit. The 19000-dalton doublet present in myosin from the red skeletal muscles was

Fig. 2. Schematic representation of the band pattern obtained on electrophoresis of partially phosphorylated rabbit cardiac-muscle myosin

Electrophoresis was done in 8M-urea-20mM-Tris-122mM-glycine, pH8.6. For explanation of abbreviations see Fig. 1.

Fig. 3. Time-course of the phosphorylation of cardiac-muscle myosin by myosin light-chain kinase

Unphosphorylated rabbit cardiac-muscle myosin (10 mg/ml) was incubated at 25°C with myosin light-chain kinase from rabbit skeletal muscle (35 µg/ml) in 0.15M-KCl-12.5 mM-magnesium acetate-5 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (1.14 µCi/µmol)-0.1 mM- CaCl_2 -0.1 mM-dithiothreitol-50 mM-Tris-40 mM-HCl, pH 7.6. Samples were removed at times indicated and reaction was stopped with 5% (w/v) trichloroacetic acid; covalently bound ^{32}P was measured in the precipitate as described in the Methods section.

partially converted into a new faster-migrating doublet on incubation with ATP and myosin light-chain kinase preparations.

Attempts to demonstrate complete phosphorylation of the light chains of cardiac- and red-muscle myosin on incubation with the light-chain kinase from white skeletal muscle, either by the change in band pattern on electrophoresis in 8M-urea, pH 8.6, or by measurement of the incorporation of ^{32}P into the protein, gave variable results. Unlike myosin from rabbit white skeletal muscle, even with excess of myosin light-chain kinase it was not possible to convert the 19000-dalton form completely into its phosphorylated form.

With all preparations of cardiac-muscle myosin the time-course of incorporation of ^{32}P on incubations under the standard conditions was unusual in that the covalently bound ^{32}P increased rapidly to a peak within 1 or 2 min, fell rapidly and then rose again to a constant value which was usually significantly less than 2 mol of P/mol of myosin (Fig. 3). This behaviour was rarely obtained with myosin from white skeletal muscle incubated with the same kinase preparation, in which case incorporation was usually linear with time and reached a plateau at values close to 2 mol of P/mol of myosin.

In general, the apparent rates of phosphorylation of cardiac-muscle myosin were low compared with those obtained with myosin from white skeletal muscle. Myosin from cardiac muscle also differed

from that of white skeletal muscle in that on incubation without added enzyme under the standard conditions for light-chain kinase assay, little formation of forms P-19K or P-19K' could be demonstrated by electrophoresis in 8M-urea, pH 8.6. Under similar conditions the 18500-dalton component of white-skeletal-muscle myosin is completely phosphorylated (Pires *et al.*, 1974).

The difference in behaviour of myosin from white skeletal and cardiac muscle could not be ascribed to the absence of myosin light-chain kinase from cardiac-muscle myosin preparations, for active kinase preparations could be obtained from freeze-dried cardiac-muscle myosin by extraction with 4 mM-EDTA, pH 7.0. It was more likely due to the action of an active myosin light-chain phosphatase which was also demonstrated in EDTA extracts of cardiac myosin. A similar phosphatase activity has been reported in extracts of white-skeletal-muscle myosin (Perry *et al.*, 1975).

Fractionation of the light chains of cardiac myosin

Attempts to fractionate the light-chain fraction of rabbit cardiac-muscle myosin by the method used by Perrie *et al.* (1973a) for the light-chain fraction of rabbit skeletal-muscle myosin were unsuccessful because of the marked tendency of the cardiac-muscle light chains to aggregate and precipitate, once separated from myosin. This aggregation resulted in the whole light-chain fraction being eluted in the void volume on gel filtration on Sephadex G-100. Fraction-

Fig. 4. Chromatography of the light-chain fraction of cardiac-muscle myosin on DEAE-Sephadex A-25

A sample (150–200 mg) of the whole light-chain fraction of rabbit cardiac-muscle myosin dissolved in 50 mM-Tris-40 mM-HCl buffer (pH 8.5)-5 M-urea-15 mM-2-mercaptoethanol, was applied to a column (90 cm × 1.5 cm) equilibrated against the same buffer. Gradient to 0.3 M-KCl-5 M-urea-50 mM-Tris-40 mM-HCl buffer (pH 7.6)-15 mM-2-mercaptoethanol was applied at point marked with arrow. Fractions (3 ml) were collected and pooled, as indicated by the bars at the top of the graph, to isolate components. ----, Unphosphorylated light-chain preparations; —, partially phosphorylated light-chain preparations; -.-.-, KCl concentration.

Fig. 5. Phosphorylation of 19000-dalton component of rabbit cardiac-muscle myosin

Light chains (10mg/ml) and myosin light-chain kinase (50 μ g/ml) were incubated at 25°C, in 12.5 mM-magnesium acetate–5 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (0.43 $\mu\text{Ci}/\mu\text{mol}$)–0.1 mM-dithiothreitol–0.1 mM- CaCl_2 –50 mM-Tris–40 mM-HCl buffer, pH7.6. Reaction was followed as for Fig. 2. ○, Whole light-chain fraction of white skeletal muscle; ●, 19000-dalton component (19K) of cardiac-muscle myosin; △, 27000-dalton light-chain component of cardiac-muscle myosin.

ation was done by chromatography in 5M-urea–50mM-Tris-HCl buffer (pH7.6)–15mM-2-mercaptoethanol on DEAE-Sephadex A-25 by a method similar to that of Katagiri & Morkin (1974). The pattern obtained depended on whether the light-chain fraction was phosphorylated or not, but peaks could be identified which corresponded to the individual light-chain components and their phosphorylated derivatives. By collecting the fractions indicated in Fig. 4 and rechromatographing them under the same conditions, peaks were obtained which, judged by electrophoresis in 8M-urea at pH8.6, were pure samples of the 27000-dalton component, the two 19000-dalton components (19K and 19K') and the phosphorylated derivatives of the latter (P-19K and P-19K') (Plate 1b).

Properties of the isolated cardiac-muscle light chains

Unlike the whole light-chain fraction, the separated 19000-dalton component did not aggregate as readily on storage in solution. Total phosphate analyses indicate that approx. 1 mol of P was present per mol of the P-19K and the P-19K' forms (Table 2). Small amounts of P were found in the 27000-dalton component and the 19K and 19K' forms of the 19000-dalton component. These were not considered signifi-

Table 3. Amino acid composition of the isolated light chains of rabbit cardiac-muscle myosin

Residues are expressed as mol/mol, based on the molecular weight determined by electrophoresis in sodium dodecyl sulphate. Results are means of duplicate analyses after 24 and 27h hydrolysis of each of two preparations (Wilkinson *et al.*, 1972). Data of Katagiri & Morkin (1974) are for the light chains of cardiac-muscle myosin of calf and are related to molecular weights of 17000 and 26000.

	19000-dalton component			27000-dalton component	
	This study	White muscle*	Katagiri & Morkin (1974)	This study	Katagiri & Morkin (1974)
Lys	11.6	(11)	16.9	22.7	30.0
His	1.6	(2)	1.37	3.3	2.7
Arg	4.5	(5)	3.5	8.4	4.8
Asx	20.1	(16)	18.6	20.1	21.1
Thr	8.4	(7)	7.5	13.1	12.7
Ser	4.1	(7)	3.2	4.8	5.6
Glx	19.2	(22)	20.7	30.8	32.9
Pro	3.92	(3)	7.7	5.0	21.7
Gly	9.4	(12)	8.9	14.5	16.7
Ala	12.4	(13)	12.0	21.3	21.8
Val	3.8	(9)	7.8	6.2	12.2
Met	6.6	(6)	6.8	8.0	10.9
Ileu	8.9	(7)	7.7	16.1	9.4
Leu	10.5	(11)	10.1	11.5	17.6
Tyr	1.6	(3)	1.6	4.3	2.8
Phe	8.6	(8)	10.1	13.0	10.5

* Values for the integral numbers of residues per mol of the 19000-dalton component from myosin of rabbit white skeletal muscle (Perrie *et al.*, 1973a).

Fig. 6. Phosphorylation of whole light-chain fractions from myosins isolated from rabbit striated muscles

Whole light-chain fraction (10 mg/ml) and rabbit myosin light-chain kinase (60 µg/ml) were incubated at 25°C with 12.5 mM-magnesium acetate-2 mM-[γ - 32 P]ATP (0.92 µCi/µmol) - 0.1 mM-CaCl₂ - 0.1 mM-dithiothreitol - 50 mM-Tris-40 mM-HCl buffer, pH 7.6. Incorporation of 32 P was followed as for Fig. 2. ○, Light-chain fraction from mixed white skeletal muscle; □, light-chain fraction from crureus or soleus muscle; ●, light-chain fraction from cardiac-muscle myosin.

cant, particularly for the 27000-dalton component, which could not be phosphorylated by myosin light-chain kinase (Fig. 5).

The amino acid analyses of the rabbit 19000- and 27000-dalton components (Table 3) were similar to those reported by Katagiri & Morkin (1974) for the light chains of myosin from calf heart.

With the isolated whole light-chain fraction from all three types of striated muscle, linear rates of incorporation of 32 P were obtained on incubation with myosin light-chain kinase under standard conditions. With the enzyme from white-skeletal-muscle myosin, the rates were similar with the light-chain fraction from red and white skeletal muscle, but the rates were significantly lower with the cardiac-muscle myosin light-chain fraction (Fig. 6). On prolonged incubation of the 19000-dalton component with excess of myosin light-chain kinase from white skeletal muscle up to 0.85 mol of P/mol of light chain was incorporated. With this enzyme a K_m value of 43.7 µM was obtained by using the cardiac-muscle light chain as substrate in the standard assay conditions (see the Methods section).

Discussion

Although there is general agreement as to the number of light chains in myosin from cardiac muscle, this is not the case with red skeletal muscle. In our view there are three different light chains of apparent mol.wt. 19000, 27000 and 29000. Under

some conditions of electrophoresis, e.g. in sodium dodecyl sulphate buffered to pH 7.0 with phosphate, the two higher-molecular-weight components are not well resolved and often migrate as a single band, giving the impression that there are only two types of light chain in red skeletal muscle (Lowey & Risby, 1971; cf. Sarkar *et al.*, 1971). Resolution is better at pH 7.0 on electrophoresis in sodium dodecyl sulphate buffered with the Tris-borate system and particularly in 8M-urea at pH 8.6, where the charge difference between the two heavier light chains produces a greater difference in mobility.

In both cardiac and red striated muscle, the 19000-dalton light-chain component was phosphorylated either by myosin light-chain kinase isolated from white skeletal muscle or by the endogenous kinase activities of the muscles.

Most of our investigations have been directed to the 19000-dalton component of cardiac muscle, and although its electrophoretic mobility appears to be very similar to that of the 19000-dalton light chain of red skeletal muscle, it cannot be concluded that these two proteins are identical until more detailed studies are made. Indeed the different rates of phosphorylation by myosin light-chain kinase of the whole light-chain fraction of red skeletal- and cardiac-muscle myosins would imply that they are not. Nevertheless, the fact that the 18000-19000-dalton light-chain component of all myosins can be phosphorylated and that these light chains can be used to restore Ca²⁺ sensitivity to molluscan myosin which has had its EDTA-extractable light chain removed (Kendrick-Jones, 1974) implies that considerable homology of function is associated with this component of the myosin molecule. Certainly, except for the serine and valine contents, the amino acid compositions of the light-chain components that are phosphorylated in myosins from white and cardiac muscles of the rabbit are very similar (Table 3). We propose in future that this component in the myosin of mammalian muscles should be referred to as the 'P-light chain' (P for phosphorylatable) rather than as the 'DTNB light chain'. In our view, this nomenclature is preferable, for phosphorylation appears to be a general property of this light chain in all vertebrate myosins so far studied (Perry *et al.*, 1975; Adelstein *et al.*, 1973). It is well known that 5,5'-dithiobis-(2-nitrobenzoic acid) does not extract the 18000-19000-dalton light component from all myosins, and the extraction is incomplete from the myosin of rabbit white skeletal muscle (Kendrick-Jones, 1974). There is as yet no evidence to suggest that phosphorylation plays a part in the ability of the light chains to restore Ca²⁺ sensitivity to myosin in the molluscan muscle. It may, however, as has been pointed out earlier (Perrie *et al.*, 1973a), play a part in the Ca²⁺ binding that has been shown to be associated with the 18500-dalton light chain of myosin (Morimoto & Harrington, 1974).

The general features of the phosphorylation of the 19000-dalton light chain of cardiac-muscle myosin are very similar to those reported for the corresponding light chain in white-skeletal-muscle myosin. One site per molecule is phosphorylated, and phosphorylation can also occur on the spontaneously modified 19000-dalton component, which presumably corresponds to the satellite form of the 18500-dalton component of myosin from white skeletal muscle of the rabbit, which is also phosphorylated (Perrie *et al.*, 1973a). This spontaneous modification to a component that migrates to the anode with higher electrophoretic mobility at pH 8.6 appears to be a property which is particularly associated with the 18000–19000-dalton light-chain component of myosins from striated muscle. Except for the myosin from rat heart, the 19000-dalton light-chain component of cardiac muscle is especially prone to this modification, the nature of which is not clear. Thiol groups are probably not involved, but an increase in net charge with an undetectable change in molecular weight could occur as a result of deamidation, or of limited proteolysis resulting in the loss of a small peptide.

With the myosin light-chain kinase from white skeletal muscle, the rate of phosphorylation of the cardiac light chain is lower than that of the corresponding white-skeletal-muscle myosin light chain. This may reflect a real difference that exists *in vivo*, for a similar difference in rate is obtained when phosphorylation is catalysed by a crude preparation of myosin light-chain kinase obtained from cardiac muscle. As with white skeletal muscle (Perrie *et al.*, 1973a), there is evidence that the 19000-dalton light chain of cardiac muscle is phosphorylated *in vivo* (McPherson *et al.*, 1974).

Of special interest is the complex time-course of phosphorylation obtained when cardiac-muscle myosin was incubated with myosin light-chain kinase. This effect probably reflects activity of the other enzymes present in the cardiac-muscle myosin preparation, such as, for example, a protein phosphatase. Although this was a consistent observation we have insufficient information about the system to decide whether it is an artifact or is of functional significance. It does, however, indicate that enzyme systems exist in muscle for rapid phosphorylation and dephosphorylation of the myosin light chain. The events appear to be related sequentially in a way that implies

interaction between the reactions involved such as might be expected in a regulatory process involving some type of feed-back control.

We are grateful to Miss Sue Brewer for skilled assistance with the amino acid analyses. We also thank Miss June Ottaway and Mr. Len Dickinson for help in the preparation of myosin light-chain kinase and myosin light chains. This work was supported in part by a grant from the Medical Research Council.

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