IDENTIFICATION OF IN VIVO PHOSPHORYLATED MYOSIN SUBUNITS

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1. Introduction

Perrie et al. [1] have shown in vitro phosphorylation of Ml_2 (DTNB) light chain [2] of rabbit skeletal muscle myosin. Myosin light chain C_2 was phosphorylated using kinases isolated from skeletal muscle myosin and [γ-^{32}P]ATP. Using similar conditions these investigators were not able to phosphorylate cardiac myosin.

The study presented here makes a comparison between in vivo incorporation of $^{32}PO_4$ into each of the cardiac myosin subunits and moles of bound phosphate present in each. These phosphorylated myosin subunits were identified by both two-dimensional gel electrophoresis and isoelectrofocusing.

2. Materials and methods

Seventy millicurie of $^{32}PO_4$ (New England Nuclear) neutralized with phosphate buffer was injected into seven pound dogs. Two days after injection, approximate peak time of protein labeling [3], the animals were sacrificed, the hearts removed and myosin purified from the cardiac ventricles as described earlier [4].

Quantification of myosin subunits and determination of specific activity are described in table 1. Various types of electrophoreses are described in figs. 1, 2 and 3. Protein determinations were made using either Lowry analyses [5] or determination of total nitrogen [6] as described in tables 1 and 2. Table 3 describes the determination of bound phosphate.

Table 1
$^{32}PO_4$ (cpm/mg protein) in purified myosin

Fractions	
Heavy chains	Light chains
2920 ± 26	1440 ± 18
($p < 0.001$)	

Average of six determinations

S.E. = $\sqrt{\Sigma x^2/n(n-1)}$

Statistical comparisons were made using Student's paired *t*-test.

For determination of isotope incorporation 50 μl samples of purified myosin fractions were precipitated with 0.5 ml of 10% trichloroacetic acid and heated at 90°C for 15 min. The pellets were washed with cold 10% trichloroacetic acid and 0.2 ml of protosol added. The mixture was heated at 55°C for 1 hr, cooled, 10 ml of scintillation fluid added, and analyzed in an automatic scintillation counter for isotope incorporation. Triplicate samples were analyzed.

For determination of total nitrogen 50 μl of sample was hydrolyzed in 25 μl of 0.9 M H_2SO_4 and heated for 45 min at 160°C. The tubes were cooled and 0.25 ml of H_2O_2 (30%) was added to each sample. The samples were then reheated at 160°C for 1 hr. The tubes were cooled and 200 λ of H_2O and 100 μl of activated Ninhydrin [6] were added, heated at 95°C for 20 min, cooled and brought to 10 ml with 50% ethanol. Nitrogen was analyzed at 570 nm against 50% ethanol blanks [6].

3. Results and discussion

Purified myosin and each of the myosin subunits are shown in fig. 1. Data for $^{32}PO_4$ incorporation into the myosin heavy and light chains are given in table 1. The in vivo labeled myosin chains were extracted

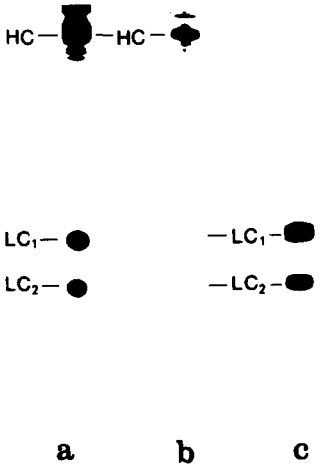
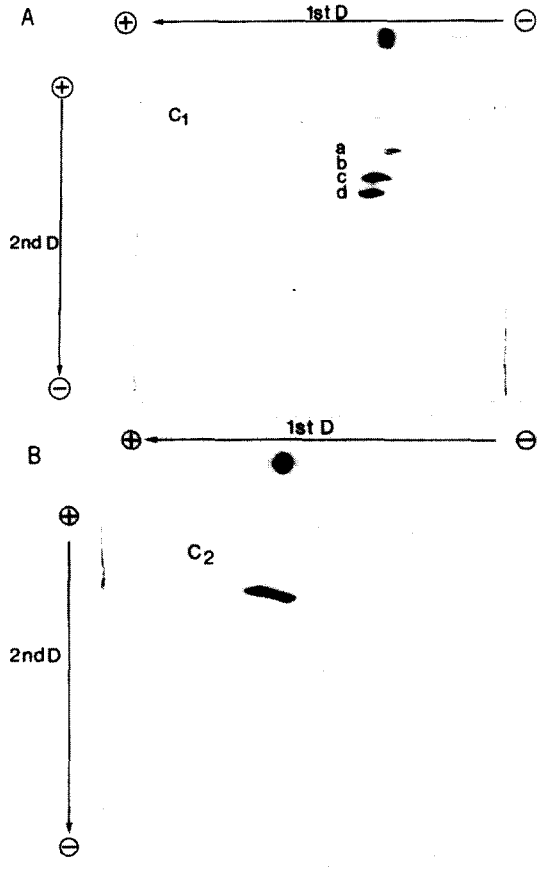


Fig. 1. Dodecylsulfate gels (6.0%) of purified myosin (a) and the purified heavy chains (b) and light chains (c) after treatment with 8 M urea [4] showing the purity of myosin and the myosin chains analyzed for specific activity.



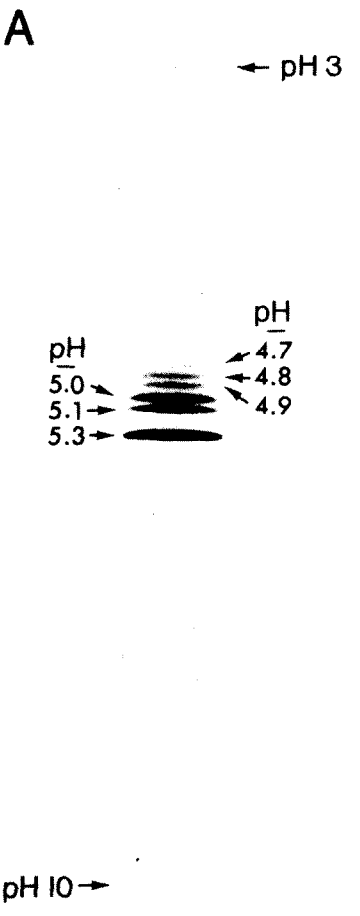
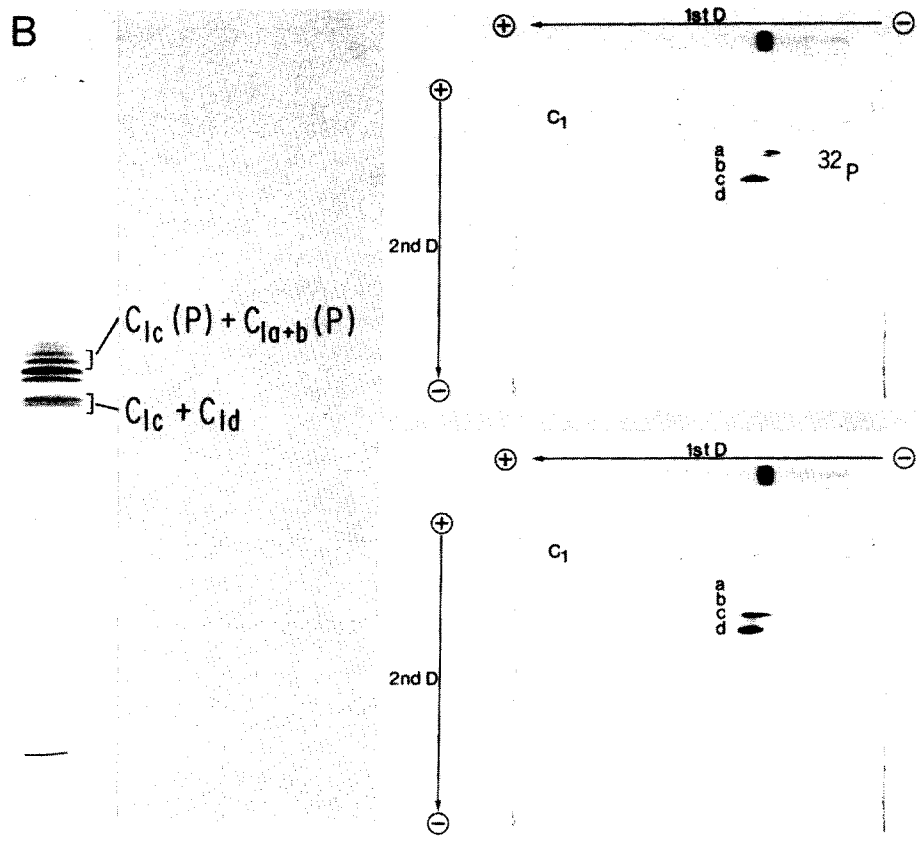


Fig. 2. The $^{32}PO_4$ labeled myosin light chains were analyzed on two-dimensional gel electrophoresis; conditons were as those described by Howard and Traut [12] except for a short dialysis as described earlier [13]. The first dimensional urea gel was electrophoresed in a boric acid buffer at pH 8.7; the running buffer was pH 8.2. The second dimensional urea gel was electrophoresed in an acetic acid buffer, pH 4.6. A stained one-dimensional urea gel is shown above the two-dimensional gel to show the point of origin. Using two comparable slab gels, 100 μg of myosin light chains were electrophoresed simultaneously on the same apparatus, but on different slabs; the stained spots were cut out of one gel and analyzed for $^{32}PO_4$ incorporation into light chains C_1 and C_2 components; the second gel was used for protein determination as described in table 2. (A) is the pattern of light chain C_1 and (B) is that for light chain C_2. The cpm/mg protein were: C_{1a+b}, 80; C_{1c}, 210; and C_{1d}, 1982.



from dodecylsulfate gels (6%) and analyzed for specific activity; a pattern of the dodecylsulfate gels used for extraction of myosin chains is shown in table 2. The $^{32}PO_4$ incorporation, expressed as cpm/mg of protein is given in table 2 and incorporation of isotope per millimole of myosin subunits is shown in table 2. A mol. wt. of 220 000 [7] was used for myosin heavy chains. The molecular weights of light chains C_1 and C_2 were found to be 28 000 and 18 500, respectively [8].

When comparing the myosin light chains, 84% of the incorporated $^{32}PO_4$, on a molar basis, was present in light chain C_2 and 16% in light chain C_1. Of the moles of bound phosphate present in the light chains (table 3), 83% was in light chain C_2 and 17% in light chain C_1. The C_1 myosin light chains were further identified by two-dimensional gel electrophoresis (fig. 2). Using this type of electrophoresis, the light chain C_1 components were resolved into isomers [13]. One of the doublets of C_{1c} was labeled as were C_{1a} and C_{1b}; there was no label in C_{1d}. Identification of phosphorylated C_1 components was determined by isoelectrofocusing (fig. 3). Fig. 3A shows the isoelectric points of each of the bands. Myosin was extracted from individual bands and analyzed on two-dimensional gels; the slower moving band on a two-dimensional urea gel was the phosphorylated one. The C_{1c} phosphorylated light chain component was partially separated from the non-phosphorylated one on two-dimensional urea gels,* however, on isoelectrofocusing they formed distinct bands which could be cut out of the gels and analyzed (fig. 3B). Identifi-

* A C_{1c} doublet is present on 2-dimensional gel electrophoresis when small protein concentrations are analyzed; large concentrations of proteins were applied to the gels in this study so that C_{1a+b} were observable.

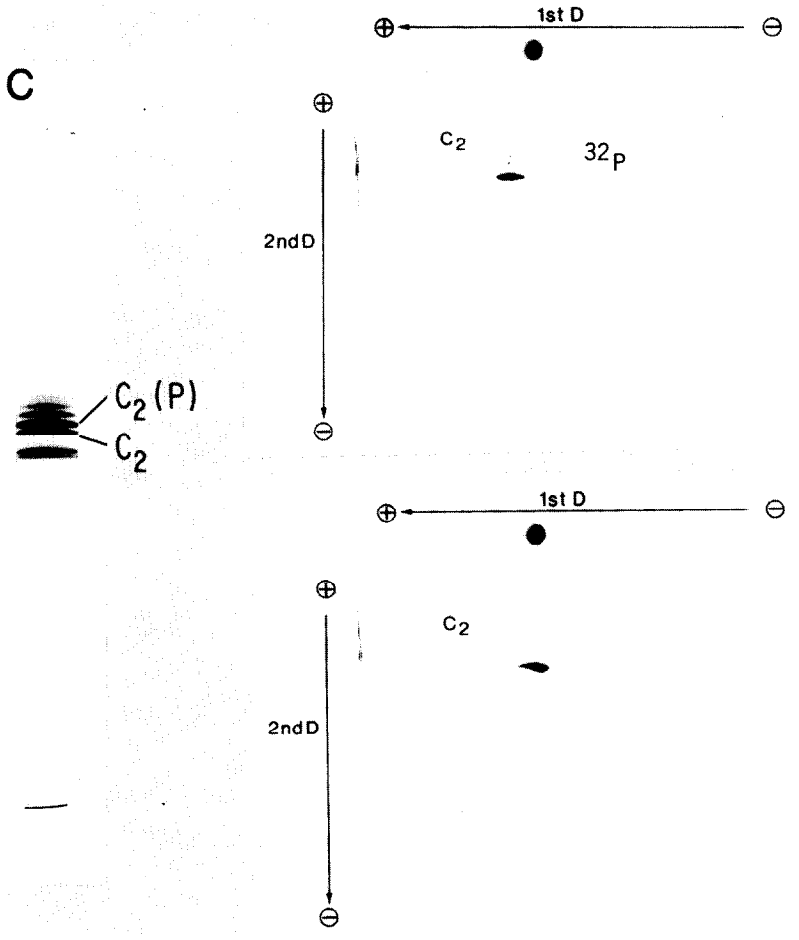


Fig. 3. (A) Isoelectrofocusing profile of canine cardiac myosin light chains. Gels contained approximately 50 μg of myosin light chains in 8 M urea; total acrylamide concentration was 5.2%; ampholine concentrations were 2%; (patterns were the same with or without 8 M urea in the gels); ampholine pH range was from 3 to 10; ammonium persulfate was 0.04%; upper reservoir (anode) contained 5% phosphoric acid; lower reservoir (cathode) contained 5% ethylene diamine. Other conditions for isoelectrofocussing are as described by Catisimpoolas [14]. The bands from several gels were cut out, pooled and electrophoresed on two-dimensional slab gels [12]. Standard myosin light chains were electrophoresed simultaneously on a comparable slab gel. Specific activity was determined on pooled bands as described in table 2 and shown in fig. 2. Identification of myosin light chain C_1 bands are shown in (B) and of light chain C_2 bands in (C).

cation of the phosphorylated and non-phosphorylated light chain C_2 components are shown in fig. 3C. The specific activity of light chain components obtained from isoelectrofocussing agreed with that obtained from extraction of proteins from two-dimensional urea gels.

The specific activity of the purified myosin heavy chains was approximately 20 times greater (cpm/mM) than the light chains, analyzing either the purified subunits (table 1) or subunits extracted from dodecylsulfate gels (table 2). The moles of bound PO_4, on the other hand, were only ten times greater in the heavy chains than in the light chains (table 3). The greater incorporation of $^{32}PO_4$ as compared to bound phosphate in the myosin heavy chains relative to the light chains may be indicative of phosphorylation of myosin occurring during protein synthesis. Earlier studies showed that the cardiac myosin heavy

Table 2
Dodecylsulfate (6%) gel of $^{32}PO_4$ labeled myosin from dog cardiac ventricles

A. (cpm/mg protein) in fractions from dodecylsulfate gels		
Heavy chains	Light chains	
	(C_1)	(C_2)
2050 ± 18	234 ± 8	2350 ± 11
(p <.001)	(p <.001)	
B. (cpm/mM of myosin subunits)		
4.5×10^8	6.5×10^6	4.2×10^7

Statistics the same as in table 1.
Data are for 12 analyses.

The bands were cut out of the gels, 0.5 ml of 30% H_2O_2 added, and heated at 55°C for 2 hr. For analyzing $^{32}PO_4$ incorporation, 10 ml of scintillation fluid was added and the samples analyzed as described in table 1. Gels were analyzed in 2 identical sets of triplicates. Isotope incorporation was determined on one set of gels and protein concentration was determined on the second set.

For determination of protein, dye was eluted with 25% pyridine (v/v) from the gels and quantitated according to standards as described earlier [9]. This technique obeyed Beer's Law for a large range of protein concentrations since the eluted dye could be diluted with increasing volumes of solvent [9]. One hundred micrograms of whole myosin or myosin light chains [4] were analyzed on a single gel. Samples were taken from various clear regions of the gel and averaged for background. Background was substracted from all analyses where isotope incorporation was determined.

Table 3
Determination of moles of bound phosphate per mole of purified myosin subunits

Heavy chains	Light chains	
	(C_1)	(C_2)
4.5 ± 0.2	0.17 ± 0.03	0.73 ± 0.05

Treatment of tissue and determination of protein is the same as in table 1
Statistics the same as in table 1.
Data were obtained from 10 separate myosin preparations.

For determination of bound phosphate, a sample of protein (< 0.07 μmoles of PO_4) was precipitated with ten volumes of 12.5% trichloroacetic acid, heated 90°C, for 15 min, and washed in ten volumes of cold 12.5% trichloroacetic acid to remove all unbound phosphate. The sample was dissolved in 100 μl of 0.1 N NaOH. (Acid washed tubes were used for all analyses); 50 μl of magnesium nitrate solution (10% Mg $(NO_3)_2$ $6H_2O$ in 95% ethanol) was added to the sample and taken to dryness over a strong flame. The sample was cooled, 0.3 ml of 0.5 N HCl was added and heated at 100°C for 15 min [10]. The sample was cooled and assayed for phosphate using the Malachite Green Reagent [11].

chains were turning over at twice the rate of the cardiac light chains [3].

Acknowledgements

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