

Identification of two forms of myosin light chain kinase in turkey gizzard

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Two forms of myosin light chain kinase from turkey gizzard are separable by ion-exchange chromatography. One is the well-characterized 130 000 M_r enzyme. Purification of the second form by affinity chromatography on calmodulin-Sepharose showed it to consist of two polypeptide chains of M_r 136 000 and 141 000. This form of the enzyme required Ca^{2+} and calmodulin for activity, was specific for the M_r 20 000 light chain of myosin, and appeared to phosphorylate the same site on the light chain as the M_r 130 000 enzyme. The low- M_r gizzard kinase may be a proteolytic fragment of a higher- M_r species or these may represent different isoenzymes.

<i>Myosin kinase</i>	<i>Phosphorylation</i>	<i>Calmodulin</i>	<i>Calcium</i>	<i>Smooth muscle</i>
		<i>Regulation</i>		

1. INTRODUCTION

Myosin light chain kinase catalyzes transfer of the terminal phosphate of ATP to a specific serine residue of the M_r 20000 light chain of smooth muscle myosin. This enzyme is believed to mediate the Ca^{2+} -induced contraction of smooth muscle, and possibly non-muscle motile systems, through its absolute dependence on calmodulin [1–3]. Myosin light chain kinases have been isolated from numerous mammalian tissues and their regulation studied [3–5]. The best studied to date is the enzyme from turkey gizzard which has been isolated as an M_r 130000 polypeptide [2,6]. During attempts to purify this enzyme we consistently observed two distinct forms of the kinase. This

paper describes these observations and the purification and partial characterization of a hitherto undescribed myosin light chain kinase of turkey gizzard.

2. MATERIALS AND METHODS

The following proteins were prepared as described: myosin [7], M_r 130000 MLCK [6], tropomyosin [8], α -actinin [9] and filamin [10] from turkey gizzard, fibronectin [11,12] from bovine blood, calmodulin [6] from bovine testes and actin [13] from rabbit skeletal muscle. Vinculin was prepared as in [14] and further purified through hydroxylapatite and Ultrogel 34. Casein, phosvitin, histone II A, histone III S, histone V S, protamine, phosphorylase *b* and phosphorylase kinase were purchased from Sigma Chemical Co. (St Louis MO). $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was obtained from New England Nuclear, $\text{ATP}\gamma\text{S}$ from Boehringer-Mannheim, and CTP and ITP from Sigma Chemical Co.

Calmodulin was coupled to Sepharose 4B as in [15]. Protein concentrations were determined by

Abbreviations: $\text{ATP}\gamma\text{S}$, adenosine 5'-O-(3-thiotriphosphate); CTP, cytidine 5'-triphosphate; DTT, dithiothreitol; EGTA, ethylene glycol bis (β -aminoethyl ether)- N,N,N',N' -tetraacetic acid; ITP, inosine 5'-triphosphate; MLCK, myosin light chain kinase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate

the biuret method [16], by the dye-binding assay [17], or spectrophotometrically in the case of pure calmodulin and M_r 130000 myosin light chain kinase: $\epsilon_{276}^{1\%} = 1.8$ [18] and $\epsilon_{278}^{1\%} = 11.4$ [2], respectively. Polyacrylamide gradient slab gel electrophoresis in the presence of 0.1% SDS was performed at 30 mA [19]. Coomassie blue-stained gels were scanned at 550 nm as in [20]. Urea-PAGE was done as in [21].

Myosin light chain kinase activity was assayed at 25°C in the presence of 25 mM Tris-HCl (pH 7.5), 0.1 mM CaCl_2 (or 1 mM EGTA), 4 mM MgCl_2 , 60 mM KCl, 15 μg calmodulin/ml, 0.5 mg myosin/ml and 0.75 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (2000–3000 cpm/nmol) as in [6].

3. RESULTS AND DISCUSSION

Myosin light chain kinase was partially purified

from frozen turkey gizzards essentially as in [2] through the Sephacryl S-300 column, with the exception that the ammonium sulfate fractionation was performed as follows: Solid ammonium sulfate was added to the Mg^{2+} extract supernatant to 60% saturation. Extraction buffer was then added to reduce the ammonium sulfate concentration to 40% saturation. After centrifugation at $15000 \times g$ for 30 min, additional solid ammonium sulfate was added to the supernatant to a final 60% saturation. The 40–60% pellet obtained by centrifugation at $15000 \times g$ for 30 min was redissolved and dialyzed prior to gel filtration essentially as in [2]. The advantage of this 'reverse' ammonium sulfate procedure is that it maximizes removal of protease activity from the kinase.

The pooled kinase-containing fractions from the Sephacryl S-300 column were dialyzed vs 2×10^1 l of 15 mM Tris-HCl (pH 7.5), 1 mM EGTA,

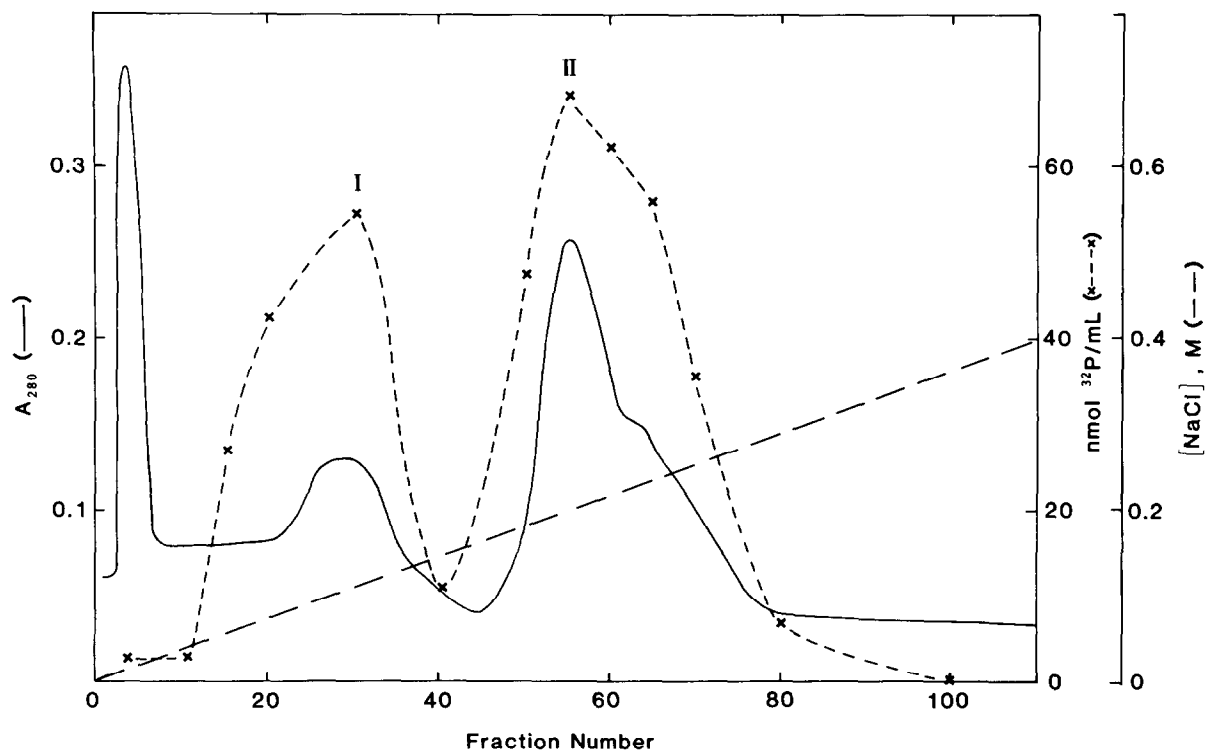


Fig.1. DEAE-Sephacel ion-exchange chromatography. The dialyzed pool from Sephacryl S-300 was applied to a column (1.4×25 cm) of DEAE-Sephacel pre-equilibrated with buffer A. Bound proteins were eluted with a linear (0–0.6 M) NaCl gradient (---) generated from 300 ml each of buffer A and buffer A containing 0.6 M NaCl. Fractions (3 ml) were collected at 20 ml/h. Aliquots (10 μl) of selected column fractions were assayed for myosin light chain kinase activity in the presence of 0.1 mM CaCl_2 (x---x) in a total reaction volume of 0.5 ml as in section 2. Assay mixtures were incubated at 25°C for 5 min.

1 mM DTT (Buffer A) and chromatographed on a column of DEAE-Sephacel (fig.1). Two peaks of myosin light chain kinase activity were eluted from the column suggesting the existence of two distinct forms of the enzyme. This possibility was corroborated by the finding that two peaks of myosin light chain kinase activity were also separated when the pooled Sephacryl S-300 fractions were chromatographed on columns of Affi-Gel blue or DEAE-Affi-Gel blue. Further purification of the peak II enzyme in fig.1 showed it to be the well-characterized M_r 130 000 enzyme [2,6]. The peak I enzyme was further purified by affinity chromatography on a column of calmodulin coupled to Sepharose 4B (fig.2). The kinase-

containing fractions eluted with EGTA were composed of a high M_r doublet (fig.3). Gel scanning revealed this doublet to be >95% pure. Densitometric scans of similar gels of 4 different kinase preparations yielded the following A:B ratios, 1.23:1, 1.03:1, 1.26:1 and 1.24:1. The two polypeptides, A and B, could not be separated under non-denaturing conditions suggesting that the peak I kinase consists of two subunits, A and B. It is possible, however, that B may be derived from A by proteolysis. A and B were determined to have M_r of 141 000 and 136 000, respectively, by SDS-polyacrylamide gradient gel electrophoresis [22]. A and B are clearly separated from the M_r 130 000 kinase by electrophoresis in the presence of

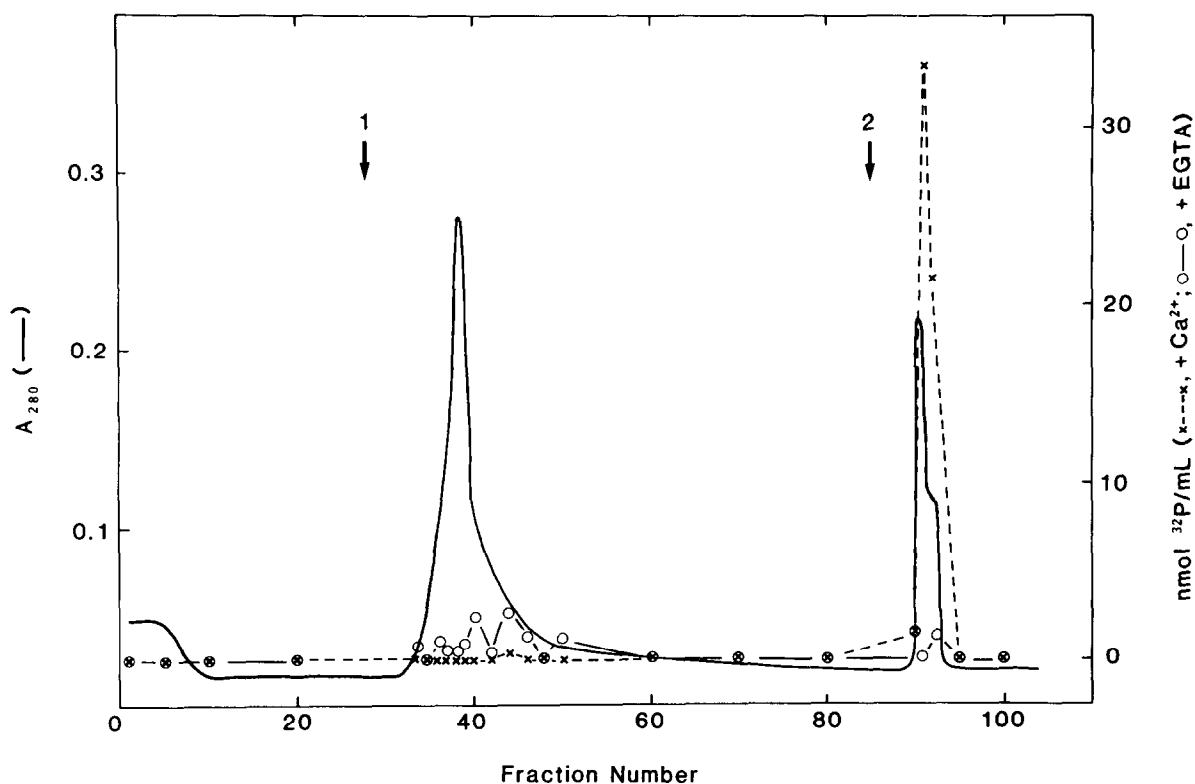


Fig.2. Calmodulin-Sepharose 4B affinity chromatography. Peak I from DEAE-Sephacel was dialyzed vs 2×10^1 of 15 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM DTT. CaCl_2 , MgCl_2 and leupeptin were added to final concentrations of 1.5 mM (i.e., 0.5 mM excess over EGTA), 1 mM and 1 mg/l, respectively, and the sample loaded on a column (1×19 cm) of calmodulin coupled to Sepharose 4B pre-equilibrated with 15 mM Tris-HCl (pH 7.5), 0.5 mM CaCl_2 , 1 mM MgCl_2 , 1 mM DTT, leupeptin (1 mg/l) (buffer B). Fractions (3.3 ml) were collected at 14.4 ml/h. After washing off excess protein with buffer B, non-specifically bound proteins were eluted by application of buffer B containing 0.2 M NaCl (arrow 1). Finally, specifically bound proteins were eluted with 15 mM Tris-HCl (pH 7.5), 2 mM EGTA, 1 mM MgCl_2 , 1 mM DTT, 0.2 M NaCl (arrow 2). Selected fractions were assayed for myosin light chain kinase activity in the presence of 0.1 mM CaCl_2 (x---x) or 1 mM EGTA (○—○) as in section 2.

Table 1

Ca²⁺, calmodulin-dependence of peak I myosin light chain kinase

Conditions	Kinase activity (mol P _i incorp./ mol myosin)
+ Ca ²⁺ + calmodulin	1.44
+ Ca ²⁺ - calmodulin	0.35
- Ca ²⁺ + calmodulin	0.09
- Ca ²⁺ - calmodulin	0.09

Peak I kinase purified through calmodulin-Sepharose 4B was incubated for 3 min at 25°C and 20 µg/ml in 25 mM Tris-HCl (pH 7.5), 4 mM MgCl₂, 60 mM KCl, 0.5 mg myosin/ml, 0.75 mM [γ -³²P]ATP (2400 cpm/nmol), and either 0.1 mM CaCl₂ (+ Ca²⁺) or 1 mM EGTA (- Ca²⁺), in the presence and absence of 15 µg calmodulin/ml, prior to quantitation of protein-bound ³²P

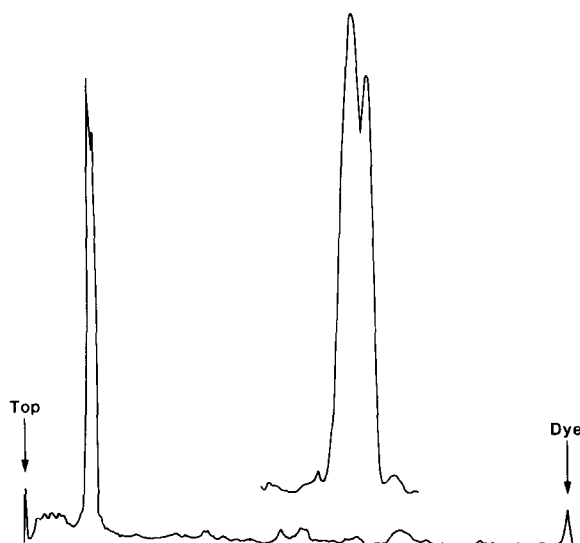


Fig.3. Purity of peak I myosin light chain kinase. An aliquot (5 µg) of the EGTA eluate from calmodulin-Sepharose 4B was electrophoresed in a 0.1% SDS, 7.5–20% polyacrylamide gradient gel. The Coomassie blue-stained gel was scanned at 550 nm. The inset shows a close-up of the scan in the region of the high *M_r* doublet to demonstrate more clearly the two bands.

SDS in either 7.5–12.5% or 5–10% polyacrylamide gradient gels.

It is clear from fig.2 that the activity of the peak

I kinase is Ca²⁺-dependent. The data in table 1 substantiate this finding and demonstrate also the calmodulin dependence of this enzyme. The low level of phosphorylation observed in the presence of Ca²⁺ and absence of added calmodulin reflects a trace of contaminating calmodulin in the myosin preparation.

Peak I kinase specifically phosphorylated the *M_r* 20000 light chain of myosin as shown by SDS-PAGE and autoradiography and also urea-PAGE followed by gel slicing and quantitation of ³²P in the gel slices (not shown). The site of phosphorylation in the *M_r* 20000 light chain (serine-19 [23]) catalyzed by *M_r* 130000 MLCK is also the site phosphorylated by the peak I kinase (fig.4): if the two kinases phosphorylated different sites on the *M_r* 20000 light chain, one would expect an increase in phosphate incorporation approaching 4 mol/mol myosin following addition of the *M_r* 130000 kinase. This is clearly not the case. Furthermore, when the two kinases were added in reverse order (i.e., *M_r* 130000 then peak I) results identical to those in fig.4 were obtained.

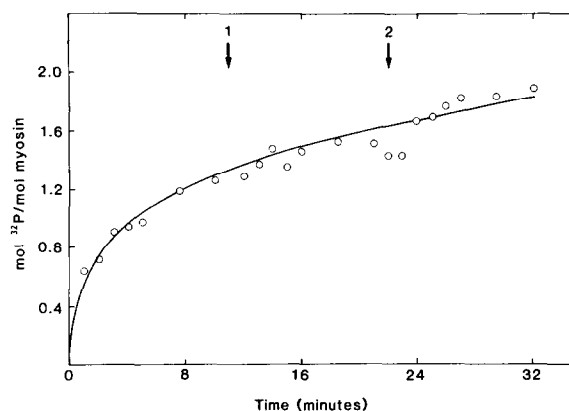


Fig.4. Sequential phosphorylation of myosin by peak I and peak II myosin light chain kinases. Purified peak I kinase (4.2 µg/ml) was incubated at 25°C in 25 mM Tris-HCl (pH 7.5), 0.1 mM CaCl₂, 4 mM MgCl₂, 60 mM KCl, 15 µg calmodulin/ml, 0.5 mg myosin/ml and 0.75 mM [γ -³²P]ATP (2300 cpm/nmol) in 11 ml total reaction vol. Aliquots (0.5 ml) were withdrawn at the indicated times for quantitation of protein-bound ³²P. At 11 min (arrow 1), additional purified peak I kinase was added to 4.2 µg/ml final conc. to ensure maximal phosphorylation by this kinase. Then at 22 min, purified *M_r* 130000 kinase was added to 4.2 µg/ml final conc.

As in the case of the M_r 130000 kinase, the peak I enzyme appears to be specific for the M_r 20000 light chain of myosin. The following proteins were examined as potential substrates of the peak I kinase and none were phosphorylated: casein, phosphorylase *b*, phosphorylase kinase, protamine, tropomyosin, α -actinin, vinculin, fibronectin, filamin, actin, histone II A, histone III S, histone V S, and protamine. Also, incubation of the peak I kinase with a crude homogenate supernatant of turkey gizzard in the presence of [γ - 32 P]ATP with and without Ca^{2+} and calmodulin, followed by SDS-PAGE and autoradiography, revealed no endogenous substrate of this kinase. Similar to earlier findings with the M_r 130000 kinase, while the ATP analog, ATP γ S, is used as a substrate by the peak I enzyme, it will not use either CTP or ITP.

The M_r 130000 myosin light chain kinase (peak II enzyme) may be derived from the peak I enzyme by partial proteolysis or it may be a distinct form of the enzyme. If there are indeed two distinct forms, elucidation of the physiological significance will lead to a fuller understanding of the regulation of smooth muscle contraction.

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