Isolation of the native form of chicken gizzard myosin light-chain kinase

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A simple and rapid procedure for the purification of the native form of chicken gizzard myosin light-chain kinase ($M_r$ 136000) is described which eliminates problems of proteolysis previously encountered. During this procedure, a calmodulin-binding protein of $M_r$ 141000, which previously co-purified with the myosin light-chain kinase, is removed and shown to be a distinct protein on the basis of lack of kinase activity, different chymotryptic peptide maps, lack of cross-reactivity with a monoclonal antibody to turkey gizzard myosin light-chain kinase, and lack of phosphorylation by the purified catalytic subunit of cyclic AMP-dependent protein kinase. This $M_r$-141000 calmodulin-binding protein is identified as caldesmon on the basis of Ca$^{2+}$-dependent interaction with calmodulin, subunit $M_r$, Ca$^{2+}$-independent interaction with skeletal-muscle F-actin, Ca$^{2+}$-dependent competition between calmodulin and F-actin for caldesmon, and tissue content.

Phosphorylation of myosin is widely believed to be a prerequisite for the contraction of smooth muscle (Adelstein & Eisenberg, 1980; Walsh & Hartshorne, 1982). This reaction is catalysed by the enzyme MLCK, which requires Ca$^{2+}$ and calmodulin for activity (Dabrowska et al., 1978; Walsh, 1981). Because of its central role in the regulation of smooth-muscle contraction, and probably also non-muscle motile processes, MLCK has been the subject of widespread interest in recent years. We previously reported the separation of two forms of MLCK from turkey gizzard (Walsh et al., 1983a), one of $M_r$ 130000 and the other a doublet of $M_r$ 141000 and 136000. The $M_r$-130000 enzyme was identical with the gizzard MLCK, which had been thoroughly characterized previously (Adelstein & Klee, 1981; Walsh et al., 1983b). The higher-$M_r$ doublet was a previously unknown form of the enzyme. The possibility arose that these may represent distinct isoenzymes of MLCK or that the $M_r$-130000 kinase may be derived from a higher-$M_r$ form by proteolysis. We have demonstrated previously, with the aid of monoclonal antibodies to the $M_r$-130000 MLCK, that this form of the enzyme is indeed derived from a higher-$M_r$ species by proteolysis and that a single form, of $M_r$ 136000, is found in the muscle (Adachi et al., 1983). The $M_r$-141000 polypeptide did not cross-react with the monoclonal antibody, suggesting it to be a distinct calmodulin-binding protein, since it co-purified with the $M_r$-136000 MLCK on calmodulin–Sepharose (Walsh et al., 1983a).

In the present paper we describe a simple and rapid procedure to purify the $M_r$-136000 MLCK to electrophoretic homogeneity, including its separation from the $M_r$-141000 polypeptide. This method eliminates the (NH$_4$)$_2$SO$_4$ fractionation used previously (Walsh et al., 1983b), thereby preventing proteolysis to the $M_r$-130000 form. The results substantiate our previous findings (Adachi et al., 1983) and indicate the $M_r$-141000 protein to be caldesmon, a major calmodulin-binding protein of smooth muscle (Sobue et al., 1981).

Materials and methods

Materials

The following proteins were purified as previously described: bovine brain calmodulin (Gopalakrishna & Anderson, 1982), $M_r$-130000 MLCK and myosin from chicken gizzard (Walsh et al., 1983b), bovine stomach MLCK (Walsh et al., 1982), skeletal myofibrils (Etlinger & Fischman, 1972) and rabbit skeletal-muscle actin (Pardee & Spudich, 1982). $\alpha$-Chymotrypsin was purchased from Boehringer–Mannheim. $M_r$ marker proteins (myosin, $\beta$-galactosidase, phosphorylase b, bovine albumin, egg albumin, glyceraldehyde-3-phos-

Abbreviations used: MLCK, myosin light-chain kinase; SDS, sodium dodecyl sulphate.

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phate dehydrogenase, carbonic anhydrase, trypsinojen, trypsin inhibitor and α-lactalbumin) were purchased from Sigma. Monoclonal antibodies to turkey gizzard M₉-130000 MLCK were prepared as previously described (Adachi et al., 1983) and were generously given by Dr. Kazuo Adachi, Department of Medical Biochemistry, University of Calgary. Calmodulin was coupled to Sepharose 4B as previously described (Walsh et al., 1982). All other materials were prepared or purchased as described previously (Adachi et al., 1983).

Comparison of peptide fragments

Limited proteolysis of MLCK (M₉, 136000) and caldesmon (M₉, 141000) was performed as follows: each protein was incubated at 0.1 mg/ml in 20 mM-Tris/HCl (pH 7.5)/1 mM-EGTA/1 mM-dithiothreitol containing α-chymotrypsin (0.2 μg/ml, i.e. [E]/[S] = 1:500, w/w) in a reaction volume of 0.25 ml. Digestion was allowed to proceed for 30 min at 25°C, and reactions were quenched by addition of an equal volume (0.25 ml) of boiling SDS-gel sample buffer. Control reactions from which α-chymotrypsin was omitted were performed simultaneously. Samples of quenched reaction mixtures were examined by SDS/5-15% polyacrylamide-gradient slab-gel electrophoresis by the method of Laemmli (1970).

Phosphorylation

Phosphorylations catalysed by the purified catalytic subunit of cyclic AMP-dependent protein kinase were performed as follows: MLCK (M₉, 136000) and caldesmon (M₉, 1410000), purified as described below, were incubated at 25°C at final concentrations of 0.15 and 0.5 mg/ml respectively in 20 mM-Tris/HCl (pH 7.5)/1 mM-EGTA/1 mM-dithiothreitol/4 mM-MgCl₂/0.2 mM-[γ-³²P]ATP with the pure catalytic subunit of bovine cardiac type-II cyclic AMP-dependent protein kinase present at a ratio of 1:100 (w/w) to protein substrate. Samples (0.2 ml) of reaction mixtures were removed at selected times and quenched by addition to 0.5 ml of 25% (v/v) trichloroacetic acid/2% (w/v) sodium pyrophosphate. Protein-bound phosphate was quantified as described by Walsh et al. (1983b). Control reactions from which the protein kinase catalytic subunit was omitted were performed simultaneously.

Caldesmon–actin interaction

Studies of the interaction between caldesmon and F-actin and the effect of calmodulin on this interaction were performed in 5 mM-Tris/HCl (pH 7.5)/0.1 mM-dithiothreitol/0.1 mM-KCl/1 mM-MgCl₂/1 mM-ATP as described by Sobue et al. (1981). The following combinations were incubated at 30°C for 30 min: (a) F-actin (0.5 mg) + 0.2 mM-CaCl₂; (b) F-actin (0.5 mg) + caldesmon (20 μg) + 0.2 mM-CaCl₂; (c) F-actin (0.5 mg) + caldesmon (20 μg) + calmodulin (10 μg) + 0.2 mM-CaCl₂; (d) F-actin (0.5 mg) + caldesmon (20 μg) + 0.2 mM-EGTA; (e) F-actin (0.5 mg) + caldesmon (20 μg) + calmodulin (10 μg) + 0.2 mM-EGTA. Mixtures were chilled on ice and centrifuged at 20 000 g for 30 min to separate actin and actin-bound proteins from soluble proteins. Supernatants and pellets were examined by SDS/7.5-20% polyacrylamide-gradient slab-gel electrophoresis.

Purification of MLCK and caldesmon

M₉-136000 MLCK and caldesmon were purified as follows. Frozen chicken gizzards were minced to yield 100 g of muscle and homogenized in 4 vol. (400 ml) of 20 mM-Tris/HCl (pH 7.5)/40 mM-NaCl/1 mM-MgCl₂/1 mM-dithiothreitol/1 mM-EGTA/0.05% Triton X-100 (buffer A) in a Waring blender for 3 × 10 s. The homogenate was centrifuged at 17 000 g for 15 min. The pellet was resuspended in buffer A without Triton X-100, homogenized and centrifuged as above. This wash step was repeated once. The resultant pellet was suspended in 4 vol. (400 ml) of 40 mM-Tris/HCl (pH 7.5)/60 mM-NaCl/25 mM-MgCl₂/1 mM-dithiothreitol/1 mM-EGTA (buffer B), homogenized and centrifuged as above. The supernatant was filtered through glass wool and loaded on a column (2.6 cm × 40 cm) of DEAE-Sephasel previously equilibrated with buffer B. The column was washed with buffer B until A₂₈₀ returned to baseline, and bound proteins were eluted with a linear gradient made from 200 ml each of buffer B and buffer B made 0.4 M in NaCl. Selected fractions were examined by SDS/7.5-20% polyacrylamide-gradient slab-gel electrophoresis. The results of this chromatography are shown in Fig. 1. Fractions were pooled as indicated.

Pool A (containing caldesmon) was dialysed overnight against 2 × 10 litres of 20 mM-Tris/HCl (pH 7.5)/0.1 M-NaCl/0.1 mM-dithiothreitol/0.2 mM-CaCl₂ (buffer C) and loaded on a column (1 cm × 8 cm) of calmodulin-Sepharose previously equilibrated with buffer C. The column was washed with buffer C and then with buffer C containing 1 M-NaCl to remove non-specifically bound proteins. Finally, calmodulin-binding proteins were eluted with 20 mM-Tris/HCl (pH 7.5)/0.1 M-NaCl/0.1 mM-dithiothreitol/1 mM-EGTA. Selected fractions were examined by SDS/7.5-20% polyacrylamide-gradient slab-gel electrophoresis. The results are shown in Fig. 2.

Pool B (containing M₉-136000 MLCK) from DEAE-Sephasel (Fig. 1) was dialysed overnight against 2 × 10 litres of 20 mM-K₂HPO₄ (pH 8.0)/1 mM-EGTA/1 mM-EDTA/1 mM-dithiothreitol/0.02% NaN₃ (buffer D) and loaded on a column
Smooth-muscle myosin light-chain kinase

**Fig. 1. Ion-exchange chromatography on DEAE-Sephacel**

The Mg$^{2+}$ extract supernatant was chromatographed as described in the Materials and methods section (flow rate = 20 ml/h; fraction size = 4 ml). Upper panel: protein was monitored by $A_{280}$ (○) and bound proteins were eluted with a NaCl gradient (----). Lower panel: selected fractions were examined by SDS/7.5–20% polyacrylamide-gradient slab-gel electrophoresis. Numbers below the gels indicate positions of gel slots (40 in all). Samples applied to gel slots are as follows (from left to right): column load at commencement of loading, column load at completion of loading, fractions 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 125, 130, 135, 140, 150, 160, 170, skeletal myofibrils ($M_r$ markers), fractions 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 214, 218, 222, 226, 230, 234, 238, 242, 246, column load. Fractions were pooled on the basis of the gel pattern as indicated by the bars: pool A = protein $a$ ($M_r = 141000$); pool B = protein $b$ ($M_r = 136000$).
Table 1. Specific activities of myosin light-chain kinases
Specific activities were determined from linear phosphorylation time course experiments obtained at very low ratios of enzyme to substrate (MLCK = 4 nM; myosin = 1.05 μM) carried out under conditions previously described (Walsh et al., 1983b) and with intact gizzard myosin as substrate. Caldesmon was assayed at concentrations up to 21 nM and found to be devoid of MLCK activity.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Specific activity (μmol of P_i/min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gizzard MLCK (M_r 130000)</td>
<td>2.9</td>
</tr>
<tr>
<td>Gizzard protein b (M_r 136000 MLCK)</td>
<td>3.3</td>
</tr>
<tr>
<td>Gizzard protein a (M_r 141000 caldesmon)</td>
<td>0.0</td>
</tr>
<tr>
<td>Bovine stomach MLCK (M_r 155000)</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Fig. 2. Affinity chromatography of protein a
Pool A from DEAE-Sepharose was dialysed and chromatographed on a column of calmodulin-Sepharose as described in the Materials and methods section. Column loading was commenced at arrow 1 of Fig. 2, and washing with buffer C at arrow 2. Buffer C containing 1 M-NaCl was applied at arrow 3, and EGTA-containing buffer at arrow 4. Lower panel: selected fractions were examined by SDS/7.5–20% polyacrylamide-gradient slab-gel electrophoresis (from left to right): pool A before dialysis, pool A after dialysis (i.e. column load), fractions 5, 10, 14, 18, 22, 26, 30, 39, 40, 41, 42, 43, 44, 55, 56, 57, 58, 59. Fractions were pooled on the basis of the gel pattern as indicated by the bar in each panel.

Results
The high-[Mg^{2+}] extract of gizzard myofibrils contains four major proteins, as previously observed (Adachi et al., 1983): filamin (M_r 240000), actin (M_r 420000) and proteins of M_r 141000 and 136000, referred to as 'a' and 'b' respectively in the gel inset of Fig. 1. These latter two proteins co-purified through calmodulin-Sepharose affinity chromatography, and the combination exhibited MLCK activity (Walsh et al., 1983a). As shown in Fig. 1, these two polypeptides can be separated by ion-exchange chromatography at 25 mM-Mg^{2+}. Under these conditions protein a did not bind to the column, whereas protein b was eluted at 0.225 M-NaCl. Protein a was then further purified by affinity chromatography on calmodulin-Sepharose (Fig. 2): protein a was eluted by chelating Ca^{2+}, indicating a Ca^{2+}-dependent interaction with calmodulin. The gel inset suggests slight proteolysis of protein a during the chromatography, a common problem encountered with calmodulin-Sepharose affinity chromatography (see below).

Protein b was purified to electrophoretic homogeneity by chromatography on a column of Affi-Gel Blue (Fig. 3). The early fractions in the protein-b peak showed evidence of very slight proteolysis (see the gel insert) and were not included in the fractions pooled for subsequent experiments. This protein also bound to calmodulin-Sepharose in a Ca^{2+}-dependent manner, but this was not exploited in its purification, since it was accompanied by significant proteolysis of protein b, apparently by a Ca^{2+}-activated protease. The procedure described here for the purification of protein b includes EGTA throughout, thereby avoiding such proteolysis. This procedure also eliminates the (NH_4)_2SO_4 fractionation shown previously to result in significant degradation (Adachi et al., 1983). Protein b was obtained with a yield of 21–31 mg/100 g of gizzard.

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Table 1 shows that protein b exhibits MLCK activity comparable with that of the $M_r$-130000 enzyme and the bovine stomach enzyme. Protein a, on the other hand, had no MLCK activity. Proteins a and b therefore appear to be distinct calmodulin-binding proteins. This was confirmed by three independent methods. Firstly, digestion of purified proteins a and b with a limiting amount of $\alpha$-chymotrypsin yielded completely different peptide fragments on SDS/5%-polyacrylamide-gradient slab-gel electrophoresis (Fig. 4): protein b was degraded to two major peptides of $M_r$ approx.
90,000 and 23,000 (lane 5), whereas protein a was almost completely digested under identical conditions, only very small amounts of several peptides being visible (lane 6). Secondly, protein b cross-reacted with a monoclonal antibody to turkey gizzard Mγ-130,000 MLCK, whereas protein a did not (Fig. 5). Co-electrophoresis of proteins a and b followed by immunoblotting showed two stained bands, but only the Mγ-136,000 protein b cross-reacted with the antibody (lane 4). Thirdly, protein b could serve as a substrate for the catalytic subunit of cyclic AMP-dependent protein kinase, a property shown previously for the Mγ-130,000 MLCK (Adelstein et al., 1978), but protein a was not phosphorylated (Fig. 6).

Protein a was identified as caldesmon, a major calmodulin-binding protein originally purified from chicken gizzard by Sobue et al. (1981), by study of its interaction with F-actin and the effect of calmodulin on this interaction (Fig. 7). The proteins used in this experiment are shown as follows: calmodulin (lanes 2 and 15), protein a (lanes 3 and 16) and actin (lanes 4 and 17). Lanes 5 and 6 indicate that, in the absence of Ca2+, caldesmon interacts with actin but not with calmodulin, since all the caldesmon is recovered in the pellet (lane 5). On the other hand, under identical conditions but in the presence of Ca2+,

![Fig. 4. Chymotryptic digestion of proteins a and b](image)

**Fig. 4. Chymotryptic digestion of proteins a and b**

Purified protein a and protein b were digested with $\alpha$-chymotrypsin as described in the Materials and methods section and compared by SDS/5-15% polyacrylamide-gradient slab-gel electrophoresis. Key to lanes (20 $\mu$g of protein per lane): 1, untreated protein b; 2, untreated protein a; 3, protein b control (no $\alpha$-chymotrypsin); 4, protein a control; 5, protein b digest; 6, protein a digest; 7, Mγ markers (205,000, 116,000, 97,400, 66,000, 45,000 and 29,000).

![Fig. 5. Immunoblotting of proteins a and b](image)

**Fig. 5. Immunoblotting of proteins a and b**

Purified protein a and protein b were electrophoresed as described in the Materials and methods section. One set of gels was stained with Coomassie Blue (A) and a duplicate set was transblotted on to nitrocellulose sheets and treated with purified monoclonal anti-MLCK and a fluorescent-labelled anti-mouse IgG (whole molecule; Sigma) to enable detection of bound antibody (B) as previously described (Adachi et al., 1983). Key to lanes: 1, Mγ markers (see legend to Fig. 4); 2, protein b; 3, protein a; 4, protein a + protein b. A control immunoblot, treated identically with the gels in the right panel, except that anti-MLCK was omitted from the soaking solution, revealed no fluorescent bands.

![Fig. 6. Phosphorylation of proteins a and b](image)

**Fig. 6. Phosphorylation of proteins a and b**

Purified protein a and protein b were treated with the catalytic subunit of cyclic AMP-dependent protein kinase (C subunit) as described in the Materials and methods section. Samples of reaction mixtures were withdrawn at the indicated times for determination of protein-bound $^{32}$P phosphate. $\square$, Protein b + C subunit; $\bigcirc$, protein a + C subunit; $\Delta$, protein a or protein b with no C subunit, i.e. controls.

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some caldesmon is recovered in the supernatant (lane 10), indicating interaction with calmodulin. Control experiments from which calmodulin was omitted indicated complete binding of caldesmon to actin in both the presence (lane 11) and the absence (lane 7) of Ca^{2+}. These results are fully consistent with the observations by Sobue et al. (1981) showing a Ca^{2+}-independent interaction between caldesmon and actin, and competition between calmodulin and actin for binding to caldesmon only in the presence of Ca^{2+}.

Discussion

A procedure is described for the purification to apparent homogeneity of M_{r}-136000 MLCK of chicken gizzard. As we have shown (Adachi et al., 1983), this represents the only form of the enzyme found in the intact smooth muscle, the M_{r}-130000 enzyme which had been characterized previously (Adelstein & Klee, 1981; Walsh et al., 1983b) being derived from this protein by proteolysis during isolation. This new procedure also enabled us to separate a M_{r}-141000 calmodulin-binding protein from the MLCK; these two polypeptides had previously co-purified (Walsh et al., 1983a), suggesting that the MLCK may be a dimer. It is now clear, however, that this M_{r}-141000 protein is a distinct protein, since it lacks MLCK activity, presents a completely different chymotryptic peptide map, does not cross-react with a monoclonal antibody to turkey gizzard MLCK, and is not a substrate of the cyclic AMP-dependent protein kinase catalytic subunit.

We have identified this calmodulin-binding protein as caldesmon, which was originally described by Sobue et al. (1981), on the basis of subunit M_{r}, Ca^{2+}-dependent interaction with calmodulin, Ca^{2+}-independent interaction with skeletal F-actin, competition between calmodulin and F-actin for binding to protein a only in the presence of Ca^{2+}, and tissue content. Sobue et al. (1981) estimated chicken gizzard caldesmon at 8% of total protein. Our estimate, based on densitometric scanning of denaturing gels of gizzard proteins solubilized in SDS-gel sample buffer, is somewhat lower than this value (3.2%). We consider that our value is probably more representative, since the conditions of extraction (0.3M-KCl) used by Sobue et al. (1981) may lead to incomplete extraction of some proteins, whereas our conditions (boiling in SDS) would be expected to effect almost quantitative solubilization of tissue proteins. In agreement with Sobue et al. (1981), the caldesmon band is clearly the major polypeptide in the M_{r} range between α-actinin (M_{r}, 102000) and myosin heavy chain (M_{r}, 205000).

On the basis also of densitometry, MLCK is present to the extent of 1.3% of total protein, which corresponds to a tissue concentration of approx. 4μM. Earlier, lower estimates of gizzard MLCK content [1.2μM (Adelstein & Klee, 1981); 1.6μM (Walsh et al., 1983b)] were due to two factors: partial proteolysis of the native M_{r}-136000 MLCK to the M_{r}-130000 fragment and other fragments of lower M_{r}, and incomplete extraction of the kinase from the myofibrillar pellet by 25mM-MgCl_{2}. We have demonstrated, using binding studies in vitro, that MLCK is associated with the thin filaments in gizzard (Dabrowska et al., 1982). Assuming an actin concentration in gizzard of 0.82mM (Hartshorne, 1981), the molar ratio of MLCK to actin is 1:205, which corresponds to approx. 2 molecules of MLCK per thin filament of 1μm length. This localization and concentration is of major importance in considering the mechanism and extent of myosin phosphorylation in the intact smooth
muscle, since it will be influenced by the degree of thick- and thin-filament overlap (Dabrowska et al., 1982).

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References
