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 136 000) is described which eliminates problems of proteolysis previously encountered. During this procedure, a calmodulin-binding protein of M_r 141 000, 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 -141 000 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 130 000 and the other a doublet of M_r 141 000 and 136 000. The M_r -130 000 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 -130 000 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 -130 000 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 136 000, is found in the muscle (Adachi *et al.*, 1983). The

M_r -141 000 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 -136 000 MLCK on calmodulin-Sepharose (Walsh *et al.*, 1983a).

In the present paper we describe a simple and rapid procedure to purify the M_r -136 000 MLCK to electrophoretic homogeneity, including its separation from the M_r -141 000 polypeptide. This method eliminates the $(\text{NH}_4)_2\text{SO}_4$ fractionation used previously (Walsh *et al.*, 1983b), thereby preventing proteolysis to the M_r -130 000 form. The results substantiate our previous findings (Adachi *et al.*, 1983) and indicate the M_r -141 000 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 -130 000 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). α -Chymotrypsin was purchased from Boehringer-Mannheim. M_r marker proteins (myosin, β -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, trypsinogen, trypsin inhibitor and α -lactalbumin) were purchased from Sigma. Monoclonal antibodies to turkey gizzard M_r -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 (M_r 136000) and caldesmon (M_r 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 μ g/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 (M_r 136000) and caldesmon (M_r 141000), 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- $[\gamma$ -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 M-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 μ g) + 0.2 mM-CaCl₂; (c) F-actin (0.5 mg) + caldesmon (20 μ g) + calmodulin (10 μ g) + 0.2 mM-CaCl₂; (d) F-actin (0.5 mg) + caldesmon (20 μ g) + 0.2 mM-EGTA; (e) F-actin (0.5 mg) + caldesmon (20 μ g) + calmodulin (10 μ g) + 0.2 mM-EGTA. Mixtures were chilled on ice and centrifuged at 200000g 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

M_r -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 $\times$ 10 s. The homogenate was centrifuged at 17000g 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 $\times$ 40 cm) of DEAE-Sephacel previously equilibrated with buffer B. The column was washed with buffer B until A_{280} 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 $\times$ 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 $\times$ 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 M_r -136000 MLCK) from DEAE-Sephacel (Fig. 1) was dialysed overnight against 2 $\times$ 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

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 = 141 000); pool B = protein b (M_r = 136 000).

(1.5 cm × 20 cm) of Affi-Gel Blue (Bio-Rad) previously equilibrated with buffer D. The column was washed with buffer D until A_{280} returned to baseline, and bound proteins were eluted with a linear gradient generated from 200 ml each of

buffer D and buffer D made 1.5 M in NaCl. Selected fractions were again examined by SDS/7.5–20%-polyacrylamide-gradient slab-gel electrophoresis. The chromatogram is depicted in Fig. 3.

Fig. 2. *Affinity chromatography of protein a*
Pool A from DEAE-Sephacel was dialysed and chromatographed on a column of calmodulin-Sephacel as described in the Materials and methods section (flow rate = 10 ml/h; fraction size = 2 ml). Upper panel: protein was monitored by A_{280} (○). Column loading was commenced at arrow 1 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 ob-

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 μ M) 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.

Protein	Specific activity (μ mol of P_i /min per mg of protein)
Gizzard MLCK (M_r 130 000)	2.9
Gizzard protein b (M_r 136 000 MLCK)	3.3
Gizzard protein a (M_r 141 000 caldesmon)	0.0
Bovine stomach MLCK (M_r 155 000)	2.8

served (Adachi *et al.*, 1983): filamin (M_r 240 000), actin (M_r 42 000) and proteins of M_r 141 000 and 136 000, referred to as 'a' and 'b' respectively in the gel inset of Fig. 1. These latter two proteins copurified through calmodulin-Sephacel 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-Sephacel (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-Sephacel 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-Sephacel 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 proteinase. 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.

Fig. 3. *Affi-Gel Blue* chromatography of protein *b*

Pool B from DEAE-Sephacel was dialysed and chromatographed on a column of *Affi-Gel Blue* as described in the Materials and methods section (flow rate = 30 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 (from left to right): column load, fraction 116, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 130, 132, 134, 136, 138, 140, 142. Fractions were pooled on the basis of the gel pattern as indicated by the bar in each panel. The position of the actin contaminant in the column load (lane 1) is indicated. This contaminant appeared in the breakthrough peak in the upper panel.

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 α -chymotrypsin yielded completely different peptide fragments on SDS/5–15%-polyacrylamide-gradient slab-gel electrophoresis (Fig. 4): protein *b* was degraded to two major peptides of M_r approx.

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

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 M_r -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 M_r -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 M_r -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 Ca^{2+} , 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 Ca^{2+} ,

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, M_r 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*. 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; $\circ$, protein *a*+C subunit; Δ , protein *a* or protein *b* with no C subunit, i.e. controls.

Fig. 7. Interaction between caldesmon and F-actin and the effect of calmodulin

The interaction between caldesmon and F-actin and the Ca^{2+} -dependence, as well as the effect of calmodulin on this interaction, were investigated as described in the Materials and methods section. Key to lanes: M_r markers [(1), values 66000, 45000, 36000, 29000, 24000, 20100 and 14200; (18), values 205000, 116000, 97400, 66000, 45000 and 29000] calmodulin, 5 μg (2 and 15); caldesmon, 3.5 μg (3 and 16); actin, 5 μg (4 and 17); actin (50 μg) + caldesmon (2 μg) + calmodulin (1 μg), no Ca^{2+} : pellet (5) and supernatant (6); actin (50 μg) + caldesmon (2 μg), no Ca^{2+} : pellet (7) and supernatant (8); actin (50 μg) + caldesmon (2 μg) + calmodulin (1 μg), + Ca^{2+} : pellet (9) and supernatant (10); actin (50 μg) + caldesmon (2 μg), + Ca^{2+} : pellet (11) and supernatant (12); actin (50 μg), + Ca^{2+} : pellet (13) and supernatant (14).

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. 4 μM . Earlier, lower estimates of gizzard MLCK content [1.2 μM (Adelstein & Klee, 1981); 1.6 μM (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-MgCl₂. 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 1 μm 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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