Purification and Characterization of Bovine Cardiac Calmodulin-dependent Myosin Light Chain Kinase*

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Myosin light chain kinase, which is located primarily in the soluble fraction of bovine myocardium, has been isolated and purified approximately 1200-fold with 16% yield by a three-step procedure. The approximate content of soluble myosin light chain kinase in heart is calculated to be 0.83 μM.

The isolated kinase is active only as a ternary complex consisting of the kinase, calmodulin, and Ca2+, the apparent Kₐ for calmodulin is 1.3 μM. The enzyme also exhibits a requirement for Mg2+ ions. Myosin light chain kinase is a monomeric enzyme with Mₙ = 85,000. The enzyme exhibits a Kₘ for ATP of 175 μM, and a Kₐₘ for the regulatory light chain of cardiac myosin of 21 μM. The optimum pH is 8.1. Kinase activity is specific for the regulatory light chain of myosin.

The specific activity of the isolated enzyme (30 nmol *P/min/mg of protein) is considerably less than the corresponding values reported for the skeletal and smooth muscle light chain kinases. This is probably due to proteolysis during extraction of the myocar- dium, a phenomenon which has, as yet, proven impossible to eliminate. In contrast to the smooth muscle enzyme (Adelstein, R. S., Conti, M. A., Hathaway, D. R., and Klee, C. B. (1978) J. Biol. Chem. 253, 8347–8350), the cardiac kinase is not phosphorylated by the catalytic subunit of cAMP-dependent protein kinase.

Regulation of vertebrate striated muscle contraction by Ca2+ ions is mediated by the troponin system (1, 2). Recent evidence from several laboratories (3–5) suggests that the mechanism of Ca2+ regulation of smooth muscle and non-muscle contractile systems, on the other hand, does not involve a troponin system, but rather occurs via Ca2+-regulated phosphorylation of one of the light chains (the regulatory or P light chain, M, approximately 20,000) of myosin. Phosphorylation of this light chain is catalyzed by a specific Ca2+-dependent kinase, referred to as myosin light chain kinase. According to this mechanism (6, 7), the kinase exists in the resting muscle as a monomer of Mₙ = 105,000 (6) or Mₙ = 125,000 (8), the regulatory light chain of myosin is in the dephosphorylated state, and actin and myosin are not interacting. An appropriate stimulus triggers an increase in cytosolic Ca2+ concentration, whereupon Ca2+ binds to calmodulin, the ubiquitous Ca2+-dependent regulator (see Ref. 9 for review). The induced conformational change in calmodulin enables it to interact with the apoenzyme. The resultant active complex (Ca2+-calmodulin-kinase) catalyzes the phosphorylation of the regulatory light chain of myosin. Myosin can then interact with actin leading to contraction. Muscle relaxation presumably occurs through hydrolysis by a specific phosphatase (10) when the Ca2+ ion concentration decreases. A similar calmodulin-dependent myosin light chain kinase has been identified in various non-muscle tissues, such as platelets and brain (11–13).

The identification of a specific kinase in red and white skeletal muscle and cardiac muscle which catalyzes the phosphorylation of the myosin regulatory light chain (14,15) raised the question of the possible presence in sarcromeric muscles of a second Ca2+-mediated regulatory system in addition to the troponin system. The possibility of a myosin-linked regulation (16, 17) was enhanced by the recent observation (18) that rabbit skeletal muscle does indeed contain a specific calmodulin-dependent myosin light chain kinase. Similarly, we have isolated and purified a calmodulin-dependent myosin light chain kinase from bovine heart.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP (300 to 400 mCi/mmol) was prepared by the method of Glynn and Chappell (19) from 32P, (New England Nuclear). Histone II A mixture, casein, phosvitin, bovine serum albumin, and ovalbumin were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Purification of Proteins—Bovine brain calmodulin was isolated and purified by a modification of the procedure described by Teo et al. (20) for the purification of bovine cardiac calmodulin, as described in detail by Walsh and Stevens (21). Glycogen phosphorylase b was prepared from rabbit skeletal muscle by the method of Fischer and Krebs (22). Glycogen phosphorylase b kinase was prepared from rabbit skeletal muscle by the method of Cohen (23). The catalytic subunit (specific activity = 1.16 μmol/min/mg) of bovine heart cAMP-dependent protein kinase type II was prepared (24) and stored (25) as previously described.

Myosin light chains were prepared from bovine cardiac, turkey gizzard, and rabbit skeletal muscle myosin according to the method of Perrie and Perry (26) by ethanol precipitation from 5 M guanidine hydrochloride. The whole light chain fraction was used as substrate of myosin light chain kinase; this fraction contained sufficient contaminating calmodulin to provide maximal activation of calmodulin-free...
Cardiac Myosin Light Chain Kinase

myosin light chain kinase, as shown by addition of extra calmodulin. In certain experiments (indicated in the text) a partially purified, calmodulin-free regulatory light chain fraction was prepared from the appropriate light chain fraction by ion exchange chromatography. The concentration of the regulatory light chain was determined by densitometric scanning at 650 nm of a Coomassie blue-stained 0.1% sodium dodecyl sulfate, 15% polyacrylamide gel. Electrophoresis of this fraction employed a 7.5% polyacrylamide cylindrical gel system described by Laemmli (33). The gel system was also used to carry out slab gel electrophoresis of the isolated kinase, gel electrophoresis employing 7.5% polyacrylamide gels was carried out in the absence of sodium azide.

Preparation of Calmodulin-Sepharose—Calmodulin was coupled to Sepharose 4B (Pharmacia Fine Chemicals Inc.) according to the method of Klee and Krinks (28), equilibrated with 40 mM Tris-HCl, pH 7.0, 10 mM 2-mercaptoethanol, 0.05 mM NaCl, 0.05 mM CaCl2, and packed into a column of 2.5 X 25 cm. The Sepharose contained approximately 1.0 mg of calmodulin/ml of packed resin. When not in use, the resin was stored in the presence of 0.05 mM CaCl2, 0.02% sodium azide.

Enzyme Assays—Myosin light chain kinase activity was measured by the amount of 32P incorporated into 0.625 mg of whole light chain fraction of bovine cardiac myosin, or 0.275 mg of partially purified regulatory light chain fraction, as indicated in the text. The assay medium contained 25 mM Tris-HCl, pH 8.0, 1.6 mM dithiothreitohol, 1.1 mM [gamma-32P]ATP (50 to 100 cpm/pmol), and either CaCl2 or EGTA. Concentrations of the reaction mixture were initiated by addition of the enzyme and incubated at 30°C for varying periods of time. Reactions were terminated by pipetting 0.06 ml of the incubation mixture onto Whatman No. 3MM filter paper (25 mu) which were then washed as described by Corbin and Reimann (39).

For assays of myosin light chain kinase activity in fractions obtained during purification of the enzyme, sodium fluoride (70 mM) was included in order to inhibit phosphatase activity; it was found that inclusion or omission of this inhibitor was without effect on the specific activity of the kinase. The initial velocity of the kinase reaction was routinely determined in the presence and absence of Ca2+ for each fraction obtained during purification by withdrawing aliquots from the reaction mixture at 1-min intervals up to 10 min incubation at 30°C. The use of short incubation times was designed to minimize possible interference due to phosphatase activity.

The ATPase activity of light chain kinase was measured by incubating the enzyme (35 pg/ml) at 30°C in 35 mM Tris-HCl, pH 8.0, 8 mM magnesium acetate, 1.6 mM dithiothreitol, 0.16 mM [gamma-32P]ATP (50 to 100 cpm/pmol), and either CaCl2 or EGTA at concentrations noted in the text. Reactions were initiated by addition of the enzyme and incubated at 30°C for varying periods of time. Reactions were terminated by pipetting 0.06 ml of the incubation mixture onto Whatman No. 3MM filter paper (25 mu) which were then washed as described by Corbin and Reimann (39).

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Purification of Bovine Cardiac Myosin Light Chain Kinase—All procedures were carried out at 4°C unless otherwise indicated. All buffers contained 0.1 mM phenylmethyalsulfonyl fluoride and 10% (v/v) glycerol.

Extraction—The ventricular muscle (1 kg) of fresh beef hearts, freed of fat and fibrous tissue, was chopped into small pieces, minced, and homogenized with 3 volumes of 40 mM Tris-HCl, pH 7.6, 10 mM 2-mercaptoethanol, or no treatment. Bovine serum albumin and the nonphosphorylated regulatory light chain of bovine cardiac myosin were used as blanks in the assay.

Gel Electrophoresis—1) Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate was carried out at 24 mA in 7.5% polyacrylamide slab gels employing the discontinuous buffer systems of Laemmli (30). 2) In order to preserve the activity of the isolated kinase, gel electrophoresis employing 7.5% polyacrylamide cylindrical gels was carried out in the absence of sodium dodecyl sulfate and in the presence of 2.5 mM dithiothreitol, using the buffer and gel system of Fairbanks et al. (34) as described by Daniel and Adelstein (35). Gels were pre-electrophoresed overnight to remove the ammonium persulfate prior to application of protein samples. Electrophoresis was performed at 2 mA/tube at 4°C. Gels were either stained and destained according to the method of Fairbanks et al. (34) and scanned at 560 nm, or cut into 2-mm slices for elution of the kinase. The gel slices were minced and incubated at 4°C overnight.

1 The abbreviations used are: EGTA, ethylene glycol bis(beta amino ethyl ether)-N,N'-tetraacetic acid; DEAE, diethylaminoethyl EDTA, ethylenediaminetetraacetic acid; TPCK, L-tosylamido-2-phenyl-ethyl chloromethyl ketone; CDR, calcium dependent regulator (calmodulin).
kinase was located in the homogenate supernatant.

**Batchwise Ion Exchange on DEAE-cellulose**—DEAE-cellulose resin (1 kg wet weight Whatman DE 52), previously equilibrated with Buffer A, was added slowly to the stirred homogenate supernatant and mixed gently for 60 min. The mixture was filtered with the aid of a Buchner funnel and washed with Buffer A containing 0.05 M NaCl. The packed resin was added slowly with stirring to 1 liter 40 mM Tris-HCl, pH 7.6, 10 mM 2-mercaptoethanol, 4 mM EDTA, 0.3 M NaCl (Buffer B), and stirred gently for 60 min. The mixture was filtered with the aid of a Buchner funnel and the resin washed twice with 500 ml of Buffer B. The 0.3 M NaCl eluate and washings were combined; this fraction contained >50% of the Ca\(^{2+}\)-dependent myosin light chain kinase activity. Solid ammonium sulfate was added up to 60% saturation. The mixture was stirred for 60 min, centrifuged at 25,000 \(\times\) g for 30 min, and the resultant pellet was resuspended in 200 ml of 40 mM Tris-HCl, pH 7.6, 10 mM 2-mercaptoethanol, 0.05 M NaCl (Buffer C), and dialyzed overnight against two changes (8 liters each) of Buffer C.

**DEAE-Sephasel Ion Exchange Chromatography**—The dialyzed sample was applied to a column (5 \(\times\) 20 cm) of DEAE-Sephasel (Pharmacia Fine Chemicals Inc.) previously equilibrated with Buffer C. The column was washed with Buffer C and eluted with a linear salt gradient generated from 1 liter each of Buffer C and Buffer C containing 0.4 M NaCl. Fig. 1 shows that a single peak of Ca\(^{2+}\)-dependent kinase activity is eluted at 0.12 M NaCl. The corresponding fractions were pooled and dialyzed overnight against two changes (8 liters each) of 40 mM Tris-HCl, pH 7.6, 10 mM 2-mercaptoethanol, 0.05 M NaCl, 0.05 M CaCl\(_2\) (Buffer D).

**Affinity Chromatography on Calmodulin-Sepharose**—The dialyzed fraction obtained after chromatography on DEAE-Sephasel was loaded onto a column of calmodulin coupled to Sepharose 4B (see "Experimental Procedures") previously equilibrated with Buffer D (Fig. 2). The column was washed with Buffer D, then with 40 mM Tris-HCl, pH 7.6, 10 mM 2-mercaptoethanol, 0.2 M NaCl, 0.05 M CaCl\(_2\) (Buffer D). Specificity bound protein was removed by washing with 40 mM Tris-HCl, pH 7.6, 10 mM 2-mercaptoethanol, 0.2 M NaCl, 0.05 M CaCl\(_2\). Specifically bound protein was eluted with 40 mM Tris-HCl, pH 7.6, 10 mM 2-mercaptoethanol, 0.2 M NaCl, 2 mM EGTA. The flow rate was 15 ml/h and 3-ml fractions were collected. Kinase activity was measured in the presence of 2 mM CaCl\(_2\) (O---O) or 2 mM EGTA (Cl----Cl) using bovine cardiac myosin whole light chain fraction (see "Experimental Procedures") as substrate. The arrows indicate the stages at which eluting buffers were changed. Fractions indicated by the bar were pooled and dialyzed against 40 mM Tris-HCl, pH 7.6, 10 mM 2-mercaptoethanol.

At -20°C in this buffer containing 30% (v/v) glycerol.

A summary of the purification is shown in Table I. Myosin light chain kinase was purified approximately 1200-fold, with a yield of 16%, which represents 6 mg of protein obtained from 1 kg of bovine ventricular muscle. This indicates a cellular content of 37.5 mg/kg or 53.5 mg/liter of intracellular water (assumed at 70% of wet weight (38)). This corresponds to a concentration of 0.63 \(\mu\)M assuming \(M_c = 85,000\) for the kinase (see below).

**Purity of the Isolated Myosin Light Chain Kinase and Molecular Weight Determination**—When the purified kinase obtained after affinity chromatography was subjected to electrophoresis under non-denaturing conditions in 7.5% polyacrylamide gels, a single strongly staining band and three faint bands were observed (Fig. 3). In order to verify that the major band seen on electrophoresis, which represents >70% of the stained protein, is indeed the myosin light chain kinase, a duplicate electrophoresis was performed, the gel was sliced, and the slices assayed for kinase activity as described under "Experimental Procedures" (Fig. 3). It is clear that the myosin light chain kinase activity co-migrates with the major protein band on the gel. Furthermore, when this major band was cut out of the nondenaturing gel and subjected to 0.1% dodecyl sulfate, 7.5% polyacrylamide gel electrophoresis, a single protein-staining band was observed with \(M_c = 85,000\) (Fig. 4).

When the purified kinase was subjected to gel filtration on a calibrated column of Sepharose 6B under nondenaturing conditions it exhibited a Stokes radius of 43.7 Å assuming a monomeric protein of \(M_c = 85,000\). A globular protein of \(M_c = 85,000\) would exhibit a Stokes radius of 39 Å (39). These data, therefore, indicate that myosin light chain kinase is an elongated monomeric protein of \(M_c = 85,000\).

**The Effect of Ca\(^{2+}\) and Calmodulin on Myosin Light Chain Kinase**—Fig. 5 demonstrates that, in the presence of a fixed amount of Ca\(^{2+}\) (0.2 mM), increasing concentrations of bovine brain calmodulin activate myosin light chain kinase. When the data are plotted according to the method of Scatchard (40), as shown in Fig. 5, the apparent \(K_d\) of the kinase for calmodulin is calculated to be \(1.3 \times 10^{-9}\) M, i.e. half-maximal.
Purification of bovine cardiac myosin light chain kinase

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*a* Determined by the method of Bradford (37).

*b* One unit is defined as that amount of enzyme which catalyzes the incorporation of 1 pmol of 32P into cardiac myosin regulatory light chain/min under the standard conditions described under "Experimental Procedures."

It is also apparent from Fig. 5 that calmodulin, in the absence of Ca2+, has no stimulatory effect whatsoever on myosin light chain kinase. It appears, therefore, that the mechanism of activation of myosin light chain kinase by calmodulin is similar to the mechanism of calmodulin-deficient bovine cardiac myosin light chain kinase, which is approximately 5 times more sensitive and operationally easier than the calmodulin-dependent cAMP phosphodiesterase assay (41). This assay has proved particularly useful for quantitating calmodulin in samples which are available in very small amounts. For example, we have used this assay to quantitate calmodulin in chick embryonic muscle extracts during muscle differentiation.2

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![Fig. 3. Gel electrophoresis and elution profile of myosin light chain kinase.](image)

Duplicate samples of myosin light chain kinase (100 μg) were electrophoresed in 7.5% polyacrylamide gels; one gel was subsequently stained with Coomassie Brilliant Blue G250, destained, and scanned at 560 nm (---); the other gel was sliced into 2 mm slices, protein eluted as indicated under "Experimental Procedures," and the eluates assayed for myosin light chain kinase activity (O- - - O).

![Fig. 4. Determination of the subunit molecular weight of myosin light chain kinase.](image)

Myosin light chain kinase (MLCK) (10 μg) and various molecular weight marker proteins (10 μg of each) were subjected to electrophoresis on 7.5% polyacrylamide slab gels in the presence of 0.1% sodium dodecyl sulfate as described under "Experimental Procedures." The marker proteins used were as follows: 1, 2, 3, 4 = α, α', β, and γ subunits, respectively, of glycogen phosphorylase b kinase; 5 = glycogen phosphorylase b; 6 = bovine serum albumin; 7 = ovalbumin.

![Fig. 5. The effect of calmodulin on the activity of bovine cardiac myosin light chain kinase.](image)

The kinase (1.2 μg/ml) was incubated for 20 min at 30°C in the presence of 35 mM Tris-HCl, pH 7.6, 8 mM magnesium acetate, 1.6 mM dithiothreitol, 2 mM [γ32P]ATP (51.5 cpm/pmol), 0.275 mg of partially purified regulatory light chain fraction of bovine cardiac myosin, and either 0.2 mM CaCl2 (O—O) or 2.4 mM EGTA (---). The amount of bovine brain calmodulin was varied as shown. Apparent Kd of calmodulin for myosin light chain kinase was determined from the reciprocal of the slope of the plot (O—O) activity in the presence of Ca2+/calmodulin) versus activity in the presence of Ca2+. Linear regression was performed by the least squares method using a minicomputer Olivetti P6060. r2 = 0.96, slope = −7.74 × 105.


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Determination of the $K_m$ Values for Substrates—Fig. 6 illustrates the results of determination of the $K_m$ of the isolated myosin light chain kinase for ATP (Fig. 6A) and the regulatory light chain of bovine cardiac myosin (Fig. 6B). The $K_m$ for ATP was calculated to be $175 \mu M$, which is comparable to the value reported for the rabbit skeletal muscle enzyme (18, 44). On the other hand, the dependence of reaction velocity on regulatory light chain concentration (shown in Fig. 6B) does not obey Michaelis-Menten kinetics, since the data do not provide a linear Lineweaver-Burk plot. Hence, a $K_m$ value for the regulatory light chain substrate cannot be calculated. However, a $K_m$ value of $21 \mu M$ is obtained from the data in Fig. 6B; this value corresponds to the $K_m$ values reported for the rabbit skeletal muscle enzyme (18, 44).

The Effect of Mg$^{2+}$ on Kinase Activity—Fig. 7 shows the effect of increasing concentrations of Mg$^{2+}$ ions on myosin light chain kinase activity assayed under optimal conditions in the presence of 1.5 mM ATP as described under "Experimental Procedures." Maximal enzymatic activity occurs at 4.5 mM Mg$^{2+}$, indicating that the enzyme requires free Mg$^{2+}$ in addition to Mg$^{2+}$ bound in the form of MgATP as substrate. Slight inhibition of kinase activity is observed at higher Mg$^{2+}$ concentrations. This may reflect competitive binding of Mg$^{2+}$ and Ca$^{2+}$ to calmodulin at relatively high Mg$^{2+}$ concentration (45), Mg$^{2+}$-calmodulin being less capable of activating the kinase than the Ca$^{2+}$-bound form of the protein. Alternatively, the kinase may exhibit another ion binding site which is inhibitory.

The Effect of Ionic Strength on Kinase Activity—The effect of increasing the ionic strength of the environment on the activity of myosin light chain kinase is shown in Fig. 8. Maximal activity is observed in the ionic strength range 0.1 to 0.25, above which the activity declines gradually. This decrease in activity at higher ionic strengths may be due to a decrease in the affinity of the kinase for calmodulin or for the regulatory light chain.

Optimum pH—The results of determination of the optimum pH of myosin light chain kinase are presented in Fig. 9. The enzyme exhibits an optimum pH of 8.1, the activity falling off quite rapidly with rise or fall in pH. The enzyme is essentially inactive below pH 5.5. Moreover, if the enzyme is maintained for a short period of time (30 to 60 min, for example) at pH 5 and then readjusted to pH 8, only about 50% of enzymatic activity is recovered.

Substrate Specificity—Various known phosphorylatable proteins, in addition to the regulatory light chain of myosin, were examined as possible substrates for the bovine cardiac myosin light chain kinase, namely casein, phosvitin, histone
be that all protein kinases have the capacity to transfer the γ-phosphoryl group from ATP to water as well as to protein substrates, albeit at a much lower rate.

We also compared the regulatory light chain of bovine cardiac myosin with the regulatory light chains of smooth muscle (turkey gizzard) and rabbit skeletal muscle as substrates of the bovine cardiac kinase. The enzyme was assayed at 3-min intervals up to 12 min, as described under "Experimental Procedures." in the presence of 0.1 mM CaCl₂ and 6 × 10⁻² m calmodulin and an excess of the appropriate regulatory light chain (10 mg/ml). Vₘₐₓ values obtained for ³²P incorporation into the various regulatory light chains were as follows: bovine cardiac muscle = 79.2 pmol/min; smooth muscle = 81.4 pmol/min; rabbit skeletal muscle = 91.7 pmol/min. Thus, the rabbit skeletal muscle regulatory light chain appears to be a slightly better substrate than the cardiac or smooth muscle proteins.

Proteolysis of Myosin Light Chain Kinase—We considered the possibility that the low specific activity of the isolated myosin light chain kinase (see above) may be due to phosphorylation of a serine or threonine residue close to either terminus of the molecule; such phosphorylation sites are known to exist in, for example, glycogen synthase (47, 48), smooth muscle myosin light chain kinase (5), cGMP-dependent protein kinase (49), and many other proteins. It should be possible, therefore, to cleave off a small phosphorylated fragment by mild proteolysis. Such treatment may be expected to relieve the inhibition due to phosphorylation, resulting in an augmentation of kinase activity which would subsequently decline upon further digestion of the molecule. As shown in Fig. 10, mild proteolysis with trypsin or chymotrypsin does not induce such an augmentation of activity but rather results in a gradual loss of kinase activity. In each case, the residual kinase activity remains Ca²⁺-dependent throughout the digestion, in contrast to the effect of mild proteolysis on calmodulin-independent cyclic nucleotide phosphodiesterases which are rendered Ca²⁺- and calmodulin-independent (50, 51).

The lack of activation upon mild proteolysis may be due to loss of the phosphorylation site during purification of the enzyme. This possibility is suggested by the relative sizes of the cardiac (M₀ = 85,000) and smooth muscle (M₀ = 125,000)

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**TABLE II**

<table>
<thead>
<tr>
<th>Substrate</th>
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<tr>
<td></td>
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<td>Light chain of bovine cardiac myosin</td>
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<td>Casein</td>
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**Fig. 9.** Determination of the optimum pH of myosin light chain kinase. The kinase (20 μg/ml) was incubated for 30 min at 30°C in the presence of 1.6 mM dithiothreitol, 8 mM magnesium acetate, 1.6 mM [γ³²P]ATP (62.5 cpm/pmol), 0.2 mM CaCl₂, 0.825 mg of whole light chain fraction of myosin, and one of the following buffers (35 mM) depending on the pH desired: piperazine, pH 5.5 to 6.0; imidazole, pH 6.1 to 7.9; Tris, pH 8.0 to 8.5.

**Fig. 10.** Mild proteolysis of myosin light chain kinase with trypsin and chymotrypsin. Myosin light chain kinase (0.1 mg/ml) of 40 mg Tri-HCl, pH 7.6, 10 mM 2-mercaptoethanol was incubated at 22°C with trypsin (C, D) or chymotrypsin (E, F) at a protease/kinase ratio of 1:100. Aliquots (0.015 ml) of reaction mixtures were withdrawn at the indicated times and reactions quenched by addition to 0.055 ml of 35 mg Tri-HCl, pH 8, 8 mM magnesium acetate, 1.6 mM dithiothreitol, 2 μM [γ³²P]ATP (52.4 cpm/pmol), 10 mg/ml of whole light chain fraction of bovine cardiac myosin, 2 μg/ml of soybean trypsin inhibitor (C, D) or 1.3 μg/ml of TPCK (E, F), and 0.1 mM CaCl₂ (C, E) or 5 mM EGTA (D, F). Quenched reaction mixtures were incubated for 30 min at 30°C at which time the amount of ³²P incorporated into the light chain substrate was determined as described under "Experimental Procedures."
Cardiac Myosin Light Chain Kinase

(8) kinases. Furthermore, we have observed that the cardiac myosin light chain kinase is highly susceptible to proteolysis during purification: if attempts are made to further purify the enzyme after affinity chromatography by gel filtration on Sepharose 6B, kinase activity is completely lost, concomitant with proteolysis of the enzyme; similarly, if the gel filtration is effected before affinity chromatography, only ~50% of the kinase activity loaded on the Sepharose 6B column is recovered, and this declines gradually to zero over a period of 3 to 4 days as a result of proteolysis.

Furthermore, we have demonstrated the presence of a protease associated with cardiac myofibrils which is capable of digesting smooth muscle (turkey gizzard) myosin light chain kinase. We have made several attempts to find extraction conditions which prevent proteolysis of cardiac myosin light chain kinase. For example, it is well known that human serum is a very effective anti-protease cocktail. When extraction of the myocardium was carried out with buffers made 1% (v/v) in human serum, no enhancement of myosin light chain kinase activity was observed in any of the fractions obtained from the tissue extract and the kinase was once more isolated as an Mr = 85,000 species.

Phosphorylation of Myosin Light Chain Kinase—It has been shown recently (8) that smooth muscle myosin light chain kinase can be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase with incorporation of 1 mol of phosphate/mol of kinase and results in a 50% diminution of activity. To determine whether or not a similar regulatory mechanism exists in the cardiac enzyme, we attempted to phosphorylate the isolated cardiac myosin light chain kinase as described under "Experimental Procedures." The reaction product was examined by 0.1% dodecyl sulfate, 7.5% polyacrylamide slab gel electrophoresis: one set of samples was subjected to autoradiography, while myosin light chain kinase bands and appropriate controls were cut out of duplicate electrophoresed samples, digested, and the radioactivity counted. The autoradiogram indicated no incorporation of 32P into the myosin light chain kinase while counting of the radioactivity in the gel slices revealed incorporation of an insignificant amount of 32P (~6.8 × 10^-4 mol/mol of kinase). Simultaneous treatment of smooth muscle (turkey gizzard) myosin light chain kinase under identical conditions, followed by autoradiography, revealed that the smooth muscle enzyme was phosphorylated by the catalytic subunit of cAMP-dependent protein kinase.

The lack of phosphorylation of the cardiac myosin light chain kinase may be due to the absence of the phosphorylation site found in the smooth muscle enzyme, probably as a result of proteolysis during purification, as suggested above. On the other hand, acid-stable phosphate, apparently resistant to phosphatase activity, is still present in variable proportions in the tissue extract and the kinase was once more isolated as an Mr = 85,000 species.

A kinase which catalyzes the phosphorylation of the Mr = 19,000 regulatory light chain of bovine cardiac myosin has been isolated and purified from bovine myocardium. Studies of the localization of myosin light chain kinase in myocardium revealed that the enzyme could be quantitatively extracted by homogenization in an EDTA-containing buffer, no kinase activity being detectable in the myofibrillar pellet, purified myofibrils, myosin, or native troponymycin fractions. This observation is somewhat at variance with the methods of isolation of myosin light chain kinase from other tissues. Thus, for example, the smooth muscle kinase has been prepared from washed myofibrils (8) or native troponymycin (6); the skeletal muscle enzyme has been isolated from washed myofibrils (18), myosin (52) or the soluble fraction obtained after homogenization of the muscle in EDTA-containing buffer (44).

The procedure described here for the purification of the bovine cardiac kinase exploits the biological affinity of the enzyme for calmodulin. The initial stages of purification, batchwise ion exchange and ion exchange chromatography in the presence of EDTA, serve to separate the apoenzyme and endogenous calmodulin, and also provide considerable purification of the apoenzyme. The major purification step, however, involves affinity chromatography on a column of calmodulin coupled to Sepharose 4B: the kinase is adsorbed quantitatively to the affinity column in the presence of Ca^2+ ions and is eluted with an EGTA-containing buffer. This indicates a Ca^2+-dependent interaction between the kinase and calmodulin similar to that known to occur in the case of various other myosin light chain kinases (8, 11, 18, 27), as well as other calmodulin-dependent enzymes such as phosphodiesteresterase and adenylate cyclase (for review, see Ref. 9). The purification procedure detailed above provides approximately a 1200-fold purification of the kinase (Table I).

Bovine cardiac myosin light chain kinase is absolutely dependent on Ca^2+ and calmodulin, the active form of the enzyme presumably being a ternary complex composed of Ca^2+, calmodulin, and the apoenzyme. The mechanism of Ca^2+ activation of the kinase presumably occurs via a mechanism similar to that regulating the activities of phosphodiesterase and adenylate cyclase (for review, see Ref. 9): at low Ca^2+ ion concentrations the kinase exists in dissociated form and is inactive. Activation is triggered by an increase in cytosolic Ca^2+ concentration whereupon Ca^2+ binds to calmodulin, thereby inducing a conformational change in the calciprotein and enabling it to interact with the kinase. The resultant ternary complex (the actual stoichiometry of which remains to be elucidated) represents the active form of the kinase. Inactivation presumably occurs via the reverse process as a result of a decrease in cytosolic Ca^2+ concentration.

Bovine cardiac myosin light chain kinase exhibits a considerable degree of substrate specificity: various known phosphorylatable proteins (casein, phosvitin, histones, phosphorylase b, and phosphorylase kinase) proved not to be substrates of the isolated kinase. A similar observation has been reported for skeletal muscle (44) and human platelet (39) myosin light chain kinases; however, Waisman et al. (53) reported a broader specificity for the skeletal muscle enzyme which may perhaps be explained by contamination with another protein kinase. The relatively high Km for ATP (175 μM) of the isolated cardiac kinase, compared with the Km values for ATP of cAMP-dependent protein kinases, appears to be characteristic of calmodulin-dependent protein kinases: rabbit skeletal muscle myosin light chain kinase has been assigned Km values for ATP of 200 to 400 μM (44) and 280 μM (18); glycogen phosphorylase b kinase (glycogen synthase kinase-II) also exhibits a high Km for ATP of 400 μM (54). Similarly, the Km for the regulatory light chain substrate of the cardiac myosin light chain kinase (21 μM) is of the same order as the Km values reported for the rabbit skeletal muscle enzyme: 24 μM (18) and 40 to 50 μM (27).

From the yield of 16% and Mr = 85,000, the concentration of myosin light chain kinase in the heart is calculated to be 0.63 μM. This is comparable to the concentration of the enzyme

3 Data submitted with this manuscript for review; available to interested readers on request.
in smooth muscle (0.57 μm) calculated from the data of Adelstein et al. (8) assuming a yield of 50%. The cardiac content of the substrate, the regulatory light chain of myosin, is 100 μm based on a content of cardiac troponin of 50 μm (55). Together with the specific activity of the isolated kinase (30 nmol 32P/min/mg of protein), these data indicate that the kinase could not catalyze the phosphorylation of the light chain substrate at a sufficient rate to constitute a mechanism of actin-myosin regulation on the time scale of a single muscle twitch, since the enzyme would catalyze the incorporation of only approximately 2 μmol of phosphate/100 μmol of regulatory light chain/min. On the other hand, the very presence of a specific phosphorylation-dephosphorylation system regulating the phosphorylation state of the regulatory light chain suggests its importance in the regulation of actin-myosin interactions.

If phosphorylation of the regulatory light chain of myosin catalyzed by calmodulin-dependent myosin light chain kinase constitutes a mechanism of regulation of cardiac muscle contraction, then the isolated enzyme must be a modified form of the native enzyme, the latter exhibiting much higher specific activity, probably in the same range as reported for the smooth (27) and skeletal (28) muscle kinases. In this context, two possible modifications to the native enzyme appear likely to account for the low specific activity of the isolated kinase: phosphorylation or proteolysis of the native myosin light chain kinase. It has been demonstrated by Adelstein et al. (8) that the smooth muscle kinase phosphorylation by the catalytic subunit of cAMP-dependent protein kinase is accompanied by an approximately 2-fold decrease in specific activity. This decrease in catalytic activity is due to a decrease in the affinity of the kinase for calmodulin as a result of phosphorylation (56). The possibility that the low specific activity of the cardiac kinase is due to the phosphorylation state of the isolated enzyme seems unlikely: while the isolated kinase contains a variable amount of phosphate which survives throughout purification of the enzyme, the enzyme binds calmodulin with high affinity (Kd = 1.3 nM) and, furthermore, the kinase exhibits a low specific activity even in the presence of saturating amounts of calmodulin.

On the other hand, the possibility that the isolated kinase is a proteolytic fragment of the native enzyme appears much more likely. We have presented evidence that the kinase is highly susceptible to proteolysis due to endogenous proteases as well as trypsin and chymotrypsin. Furthermore, we have demonstrated that bovine myocardium contains a protease which digests turkey gizzard myosin light chain kinase. In addition, comparison of the trypsin peptides generated by limited digestion of the smooth muscle and cardiac kinases by the method of Cleveland et al. (57) reveals the presence of many common peptides, there being more peptides derived from the heavier smooth muscle enzyme.
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Purification and characterization of bovine cardiac calmodulin-dependent myosin light chain kinase.
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