Bovine Stomach Myosin Light Chain Kinase: Purification, Characterization, and Comparison with the Turkey Gizzard Enzyme†

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ABSTRACT: Myosin light chain kinase has been purified from bovine rumen by a simple and rapid procedure involving extraction from washed "myofibrils" with a high Mg^{2+} concentration, ammonium sulfate fractionation, Affi-Gel Blue chromatography, and ion-exchange chromatography. A similar method was successful in purification of the kinase from turkey gizzard. The bovine stomach enzyme exhibits many properties in common with the turkey gizzard enzyme: kinase activity is dependent on Ca^{2+} and calmodulin, with an apparent K_d for calmodulin of 1.3×10^{-9} M; bovine stomach myosin light chain kinase exhibits Ca^{2+}-dependent interaction with immobilized calmodulin; bovine stomach and turkey gizzard kinases appear to catalyze phosphorylation of the same site on the 20 000-dalton light chain of smooth muscle myosin; and bovine stomach myosin light chain kinase is a substrate of the cAMP-dependent protein kinase with phosphorylation of two sites in the apoenzyme and one site in the holoenzyme (calmodulin–kinase complex). The rate of phosphorylation of the bovine stomach enzyme is significantly faster than that of the turkey gizzard enzyme in both the presence and absence of bound calmodulin. As in the case of turkey gizzard myosin light chain kinase, phosphorylation of both sites on the bovine stomach enzyme by cAMP-dependent protein kinase reduces the affinity of the kinase for calmodulin. The amino acid composition of bovine stomach myosin light chain kinase is very similar to that of the turkey gizzard and chicken gizzard enzyme. The major difference between turkey gizzard and bovine stomach myosin light chain kinases is the molecular weight of the apoenzyme: the turkey gizzard enzyme has M_r 130 000 and the bovine stomach enzyme has M_r 155 000 as determined by 0.1% dodecyl sulfate–7.5–20% polyacrylamide gradient gel electrophoresis.

The myosin molecule is a hexamer consisting of two heavy chains (M_r ~200 000) and two pairs of light chains of varying molecular weights (16 000–27 000) depending on the source of the myosin. Myosin light chain kinase (MLCK)[1] catalyzes the transfer of the γ-phosphate of ATP to one pair of light chains. The enzyme is composed of two components: an apoenzyme or catalytic subunit and a regulatory subunit which was shown to be calmodulin (the ubiquitous Ca^{2+}-binding regulatory protein) by Dabrowska et al. (1978) for the smooth muscle kinase and by Yazawa et al. (1978) for the skeletal muscle kinase. Although MLCK is widely distributed, the role of myosin phosphorylation in most tissues is not established, with the possible exception of smooth muscle. In the case of smooth muscle myosin the 20 000-dalton light chains are phosphorylated, and the specific residue involved has been identified as serine-19 (Maita et al., 1981). It is frequently suggested that the phosphorylated state of smooth muscle myosin dictates contractile activity; phosphorylated myosin can interact with actin, hydrolyze ATP, and result in shortening and the development of tension. On the other hand, dephosphorylated myosin is dormant and is not involved in cross-bridge cycling. The dephosphorylated state would, therefore, be expected in relaxed muscle. Dephosphorylation of myosin is achieved by one or more phosphatases (Pato & Adelstein, 1980). In the simplest interpretation of this scheme the only regulatory factors are those which control the phosphorylation level of myosin light chains, namely, the kinase and the phosphatase. The involvement of an additional process was subsequently suggested, and it was shown that phosphorylation of the relevant site on the MLCK apoenzyme by the cAMP-dependent protein kinase reduces its affinity for calmodulin (Conti & Adelstein, 1981).

MLCK has been isolated, or partially purified, from numerous mammalian tissues including rabbit skeletal muscle (Pires & Perry, 1977; Yazawa & Yagi, 1978; Nairn & Perry, 1979; Blumenthal & Stull, 1980; Edelman & Krebs, 1981), chicken gizzard (Dabrowska et al., 1977; Uchiwa et al., 1982), turkey gizzard (Adelstein et al., 1978; Adelstein & Klee, 1981), bovine cardiac muscle (Walsh et al., 1979; Wolf & Hofman, 1980), bovine aorta (Vallet et al., 1981; DiSalvo et al., 1981), platelets (Daniel & Adelstein, 1976; Dabrowska & Hartshorne, 1978; Hathaway & Adelstein, 1979), and brain (Dabrowska & Hartshorne, 1978; Hathaway et al., 1981). A variable feature of the various MLCK preparations is the size of the apoenzyme (Walsh & Hartshorne, 1982). It has been suggested (Walsh et al., 1979; Walsh & Guilleux, 1981) that these variations could be due to differing degrees of proteolysis and that the native enzymes for each tissue are structurally homologous and subject to similar modes of regulation. At the moment the native form of the smooth muscle MLCK has not been clearly identified, although most studies are carried out with an apoenzyme of M_r 125 000–130 000 (Aldelstein et al., 1978). Using a modified procedure, we have isolated a MLCK of M_r ~155 000 from bovine rumen. Since it is possible that the higher molecular weight form of the MLCK reflects more closely the native enzyme, we felt it was important to establish some of its properties.

Materials and Methods

[γ-^{32}P]ATP was obtained from New England Nuclear. Myosin was purified from turkey gizzard and bovine stomach

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[1] Abbreviations: CaM, calmodulin; cAMP, cyclic adenosine 3′,5′-phosphate; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MLCK, myosin light chain kinase; $NaDodSO_4$, sodium dodecyl sulfate; $NaPP_i$, sodium pyrophosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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in the fully dephosphorylated form and free of myosin light chain kinase activity according to Persechini & Hartshorne (1981). The catalytic subunit of bovine cardiac cAMP-dependent protein kinase type II was purified as described by Demaille et al. (1977). Turkey gizzard MLCK was prepared by a modification of the method described below for the bovine stomach enzyme (Walsh et al., 1982a). Calmodulin was purified from bull or ram testes by a modification of the procedure of Wallace et al. (1980). Frozen testes (~250 g) were freed of their peripheral connective tissue layer, chopped, and homogenized in 3 volumes of 25 mM Tris-HCl (pH 7.5), 1 mM EGTA, and 0.02% NaN_3. The homogenate was centrifuged at 10000*g* for 60 min, and the supernatant was filtered through glass wool and centrifuged at 150000*g* for 90 min. The supernatant was again filtered through glass wool and $CaCl_2$ (1 M) added to a final concentration of 2 mM. The sample was applied (at a flow rate of ~20 mL/h) to a column (1.5 × 30 cm) of 2-chloro-10-(3-aminopropyl)phenothiazine coupled to Sepharose 4B as described by Jamieson & Vanaman (1979) and previously equilibrated with 25 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$, and 0.02% NaN_3. The column was washed with this buffer, and nonspecifically bound proteins were eluted by washing for at least 12 h with the same buffer containing 0.5 M NaCl. Calmodulin was then eluted with 25 mM Tris-HCl (pH 7.5), 10 mM EGTA, 0.5 M NaCl, and 0.02% NaN_3, dialyzed overnight vs. 2 × 10 L of 10 mM NH_4HCO_3 (pH 8.0), and lyophilized. Calmodulin prepared in this way was electrophoretically homogeneous, and the yield was determined to be ~320 mg/kg of testes. The phenothiazine–Sepharose column was regenerated by washing with several column volumes of 6 M guanidine hydrochloride followed by 25 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$, and 0.02% NaN_3.

Calmodulin was coupled to cyanogen bromide activated Sepharose 4B (Sigma Chemical Co., St. Louis, MO) essentially by the method of March et al. (1974). The resin was equilibrated with 15 mM Tris-HCl (pH 7.5), 0.2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM DTT, and 0.02% NaN_3 and packed in a column (1 × 19 cm). The amount of bound calmodulin was determined to be 0.9 mg/mL packed resin. After four to six uses, the column was washed with several column volumes of 6 M guanidine hydrochloride followed by equilibration buffer.

Protein concentrations were determined by the biuret method (Itzhaki & Gill, 1964) or the dye-binding assay as described by Spector (1978). Concentrations of pure turkey gizzard myosin light chain kinase and calmodulin were determined by absorbance measurements using the following molar extinction coefficients: $\epsilon_{278}^{1\%} = 11.4$ for MLCK (Adelstein & Klee, 1981) and $\epsilon_{276}^{1\%} = 1.8$ for calmodulin (Watterson et al., 1976).

Electrophoresis was performed on 7.5–20% polyacrylamide gradient slab gels in the presence of 0.1% $NaDodSO_4$ at 30 mA by using the discontinuous buffer system of Laemmli (1970). Gels were stained with Coomassie blue R-250 (0.06% in 45% ethanol, 10% acetic acid) and destained with 10% acetic acid. Gels were scanned at 550 nm on a Zeiss PM6 spektralphotometer attached to a Spectra-Physics SP 4050 printer/plotter, an SP 4020 data interface, and an SP 4000 central processor. Molecular weight values were calculated as described by Lambin (1978). The molecular weight standards used were myosin heavy chain (M_r ~200000), turkey gizzard MLCK (M_r ~130000), glycogen phosphorylase *b* (M_r ~96000), bovine serum albumin (M_r ~68000), ovalbumin (M_r ~43000), the 20000- and 17000-dalton light chains of smooth muscle myosin, myoglobin (M_r ~16900), and cytochrome *c* (M_r ~13400).

Phosphorylation Assays. Phosphate (^{32}P) incorporation into the 20000-dalton light chain of turkey gizzard or bovine stomach myosin catalyzed by MLCK and ^{32}P incorporation into MLCKs catalyzed by the catalytic subunit of cAMP-dependent protein kinase were measured as described by Mrwa & Hartshorne (1980). Experimental details are provided in the appropriate figure legends. Specific activities of MLCK fractions were obtained at very low ratios of enzyme to substrate (0.32 nM MLCK:1.05 μM myosin). Time courses of myosin phosphorylation, up to 10 min, were determined, and initial velocities of phosphorylation were calculated. The initial phases of phosphorylation (approximately 0–3 min) were usually linear, provided that the myosin used as substrate was not prephosphorylated.

Amino Acid Analyses. Turkey gizzard and bovine stomach MLCKs (9 nmol) were dialyzed overnight vs. two changes (10 L each) of distilled, deionized water. Aliquots (3 nmol) were transferred to hydrolysis tubes and hydrolyzed in 6 N HCl at 110 °C for 22, 48, and 72 h. Amino acid analyses were performed on a Beckman Model 118 amino acid analyzer. Cysteine was determined as carboxymethylcysteine following alkylation of each protein (3 nmol) with iodoacetic acid as previously described (Flink et al., 1979).

Results

Purification of Bovine Stomach Myosin Light Chain Kinase. The muscle layers of bovine stomach (specifically the rumen) were dissected free of the stomach lining and connective tissue layers and either stored frozen at −20 °C until use or treated immediately as follows.

Homogenization. The muscle was minced to yield 250 g, homogenized in a Waring blender at top speed for 3 × 5 s in 4 volumes of 20 mM Tris-HCl (pH 7.5), 40 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, and 0.05% (v/v) Triton X-100 (buffer A) and centrifuged at 15000*g* for 15 min. The pellet was suspended in 4 volumes of buffer A lacking Triton X-100 by homogenization and centrifuged as before. The pellet was resuspended in 4 volumes of buffer A lacking Triton X-100, homogenized, and centrifuged as before.

Kinase Extraction. The final washed pellet was suspended in 4 volumes of 40 mM Tris-HCl (pH 7.5), 60 mM KCl, 25 mM $MgCl_2$, 1 mM EGTA, and 1 mM DTT (buffer B), homogenized in a Waring blender at top speed for 3 × 5 s, and centrifuged at 15000*g* for 30 min.

Reverse Ammonium Sulfate Fractionation.[2] The supernatant was filtered through glass wool, and solid ammonium sulfate was added slowly with stirring to 60% saturation (363 g/L). Buffer B was added slowly with stirring to reduce the ammonium sulfate concentration to 40% saturation (226 g/L), and the mixture was centrifuged at 15000*g* for 30 min. The supernatant was filtered through glass wool and additional ammonium sulfate (123 g/L) added to a final concentration of 60% saturation. The mixture was centrifuged at 15000*g* for 30 min.

Affi-Gel Blue Chromatography. The 40–60% ammonium sulfate pellet was suspended in ~50 mL of 20 mM K_2HPO_4 (pH 8.0), 1 mM EGTA, 1 mM EDTA, 1 mM DTT, and 0.02% NaN_3 (buffer C) with the aid of a hand-operated glass/glass homogenizer and dialyzed overnight against buffer C (2 × 10 L). The dialyzed sample was applied to a column of Affi-Gel Blue and eluted with a linear NaCl gradient, as

[2] Use of the reverse ammonium sulfate fractionation procedure was suggested to us by Dr. S. Ebashi, University of Tokyo.

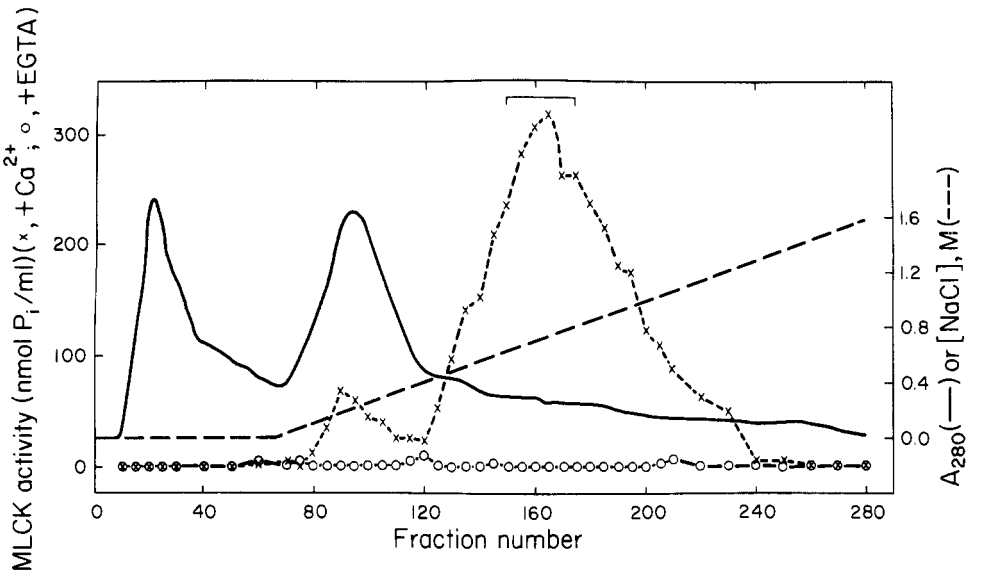

FIGURE 1: Affi-Gel Blue chromatography of the dialyzed 40–60% ammonium sulfate fraction. The dialyzed sample was loaded on a column (1.4 × 25 cm) of Affi-Gel Blue previously equilibrated with buffer C. Excess protein was washed off the column with buffer C and bound proteins eluted with a linear NaCl gradient (0–1.5 M) made from 200 mL each of buffer C and buffer C containing 1.5 M NaCl. MLCK activity was assayed in the presence of 0.1 mM $CaCl_2$ (×) or 1 mM EGTA (○) as described under Materials and Methods. Fractions (3.3 mL) were collected at a flow rate of 20 mL/h. The peak of MLCK activity eluted at 0.72 M NaCl. Fractions indicated by the bar were pooled and dialyzed prior to ion-exchange chromatography.

Table I: Purification of Bovine Stomach Myosin Light Chain Kinase[a]

purification step	total volume (mL)	protein concn[b] (mg/mL)	total protein (mg)	sp act. (μmol min^{-1} mg^{-1})	total act. (μmol/min)	yield (%)	purification (-fold)
Mg^{2+} extract supernatant	1040	2.01	2090.4	0.093	194.4	100	
40–60% ammonium sulfate	56	7.69	430.6	0.248	106.8	54.9	2.7
Affi-Gel Blue	87	1.16	100.9	0.92	92.8	47.7	9.9
DEAE-Sephacel	40	0.10	4.0	8.10	32.4	16.7	87.1

[a] Starting material: 250 g of bovine stomach smooth muscle. [b] Determined by the biuret method (Itzhaki & Gill, 1964) or according to Spector (1978).

described in the legend to Figure 1. Selected fractions were assayed for MLCK activity in the presence and absence of Ca^{2+}. Fractions were pooled as indicated by the bar in Figure 1 and dialyzed overnight against 2 × 10 L of 20 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, and 1 mM DTT (buffer D). The MLCK from bovine stomach was eluted at approximately 0.7 M NaCl, whereas the gizzard enzyme was eluted at approximately 0.4 M NaCl. The small peak of Ca^{2+}-dependent kinase activity which eluted at ~0.2 M NaCl with a large protein peak was not studied further.

DEAE-Sephacel Ion-Exchange Chromatography. The dialyzed sample was applied to a column of DEAE-Sephacel previously equilibrated with buffer D and elution achieved with a linear NaCl gradient as described in the legend to Figure 2. Selected fractions were again assayed for MLCK activity in the presence and absence of Ca^{2+}. Fractions were pooled as indicated by the bar in Figure 2, made 5% (w/v) in sucrose, and stored at −20 °C in small aliquots (0.5–1.0 mL) in plastic tubes.

A summary of a typical purification is shown in Table I. Considerable purification is achieved prior to the Mg^{2+} extraction due to almost quantitative removal of all soluble proteins, but accurate quantitation of MLCK activity becomes feasible only after its extraction from actomyosin. Myosin light chain kinase was purified approximately 90-fold from the Mg^{2+} extract supernatant, with a yield of 17%, which represents 4 mg of protein obtained from 250 g of bovine stomach muscle. This gives a cellular content of 96 mg/kg or 120 mg/L intracellular water (assumed to be 80% of wet weight) which corresponds to a concentration of 0.8 μM, assuming M_r 155000 (see below). This value is an underestimate since the kinase is not quantitatively extracted from washed myofibrils with a single high [Mg^{2+}] wash and compares with a tissue content of 1.6 μM determined for turkey gizzard MLCK (Walsh et al., 1982a).

Table II: Comparison of Specific Activities of MLCKs

substrate	sp act. (μmol min^{-1} mg^{-1})[a] stomach kinase	gizzard kinase
stomach myosin	8.9	6.1
gizzard myosin	8.1	8.7

[a] Activities were calculated from the initial phase of the phosphorylation reaction (see Materials and Methods).

Shown in Table II are the specific activities of the purified MLCKs from bovine stomach and turkey gizzard, using stomach and gizzard myosins as substrate. The activities are comparable, and in each case the MLCK shows a slight preference for its native substrate.

Purity and Molecular Weight Determinations. Figure 2 illustrates gel electrophoretic patterns of the major fractions obtained during the MLCK purification and compares the final product with turkey gizzard MLCK (M_r 130000). Clearly bovine stomach MLCK (lane D; >92% pure as determined by densitometric scans of similar gels of the purified enzyme) has a higher molecular weight than the gizzard enzyme (lane F): two discrete bands are apparent when the two kinases are

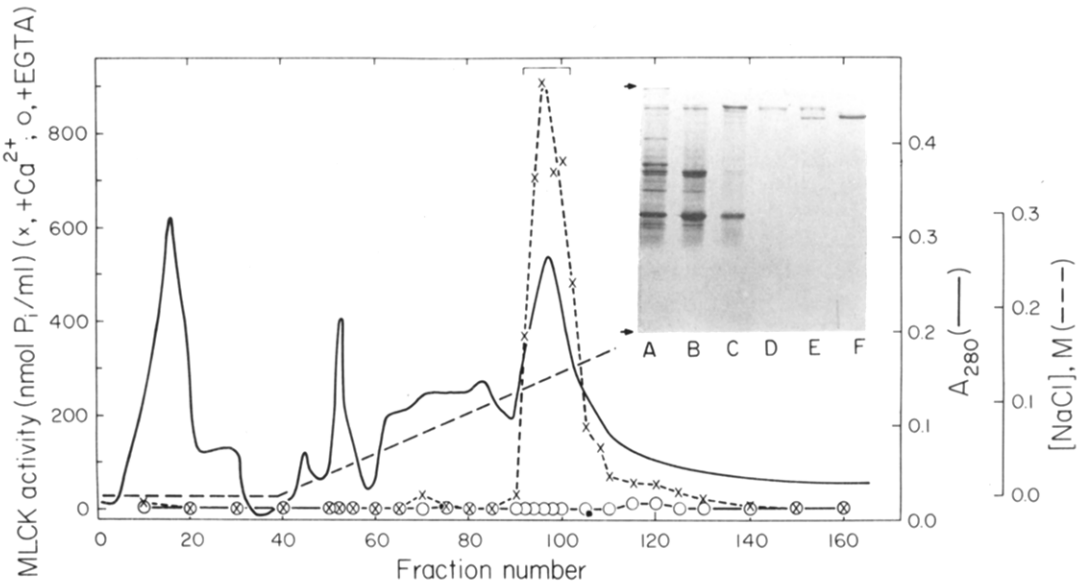

FIGURE 2: DEAE-Sephacel ion-exchange chromatography. The dialyzed eluate from the Affi-Gel Blue column was loaded on a column (1.4 × 25 cm) of DEAE-Sephacel previously equilibrated with buffer D. Excess protein was washed off the column with buffer D and bound proteins eluted with a linear NaCl gradient (0–0.3 M) made from 250 mL each of buffer D and buffer D containing 0.3 M NaCl. MLCK activity was assayed in the presence of 0.1 mM $CaCl_2$ (×) or 1 mM EGTA (o). Fractions (3.6 mL) were collected at a flow rate of 20 mL/h. The peak of MLCK activity eluted at 0.15 M NaCl. Fractions indicated by the bar were pooled and stored as described in the text. The inset represents Coomassie blue stained gels of the following samples: (A) Mg^{2+} extract supernatant (20 μg), (B) dialyzed 40–60% ammonium sulfate fraction (20 μg), (C) Affi-Gel Blue column eluate (20 μg), (D) DEAE-Sephacel column eluate, i.e., purified bovine stomach MLCK (5 μg), (E) bovine stomach MLCK (5 μg) plus turkey gizzard MLCK (5 μg), and (F) turkey gizzard MLCK (10 μg). The top arrow indicates the origin of the gel and the bottom arrow the position of the marker dye.

coelectrophoresed (lane E). A molecular weight of 155 000 was determined for the bovine stomach enzyme by 0.1% $NaDodSO_4$–7.5–20% polyacrylamide gradient gel electrophoresis. The purity of the kinase was not assessed under nondenaturing conditions.

Amino Acid Composition. Table III shows the amino acid compositions determined for the turkey gizzard and bovine stomach MLCKs. The amino acid composition of the turkey gizzard kinase is very similar to that reported by Adelstein & Klee (1981) which is included in Table III for comparison. The composition of the bovine stomach enzyme closely resembles that of the turkey gizzard enzyme, bovine stomach MLCK having slightly larger amounts of almost all residues, reflecting the higher molecular weight. The only exceptions to this statement are methionine and tyrosine residues which are slightly less abundant in bovine stomach MLCK probably reflecting differences in the sequences of the two kinases. Particularly noteworthy are significantly higher contents of the uncharged residues threonine, serine, proline, glycine, and leucine in the bovine stomach MLCK compared with turkey gizzard MLCK. As discussed below, if the 130 000-dalton MLCK of turkey gizzard is a proteolytic fragment of a parent enzyme of 155 000 daltons which is structurally homologous to the bovine stomach enzyme, the missing 25 000-dalton peptide would presumably be rich in these residues.

Ca^{2+} and Calmodulin Dependence. Bovine stomach MLCK exhibits a requirement for Ca^{2+} and calmodulin (Figure 3). The enzyme is inactive in the absence of Ca^{2+} (presence of EGTA). The very low rate of myosin phosphorylation observed in the presence of Ca^{2+} but absence of added calmodulin (Figure 3) is due to a trace of contaminating calmodulin in the myosin preparation. This conclusion was confirmed by the observation that a similar phosphorylation rate occurred when the bovine stomach MLCK was replaced by turkey gizzard MLCK in the presence of Ca^{2+} without addition of exogenous calmodulin (data not shown). The apparent K_d of the kinase for calmodulin was determined to be (1.26 ± 0.04) × 10^{-9} M. The interaction between bovine stomach MLCK and calmodulin was demonstrated by the observation that the stomach kinase binds to a calmodulin–Sepharose affinity column in the presence of Ca^{2+} and is eluted by chelation of the calcium ions with EGTA.

Table III: Amino Acid Composition of Bovine Stomach and Turkey Gizzard MLCKs[a]

residue	turkey gizzard	turkey gizzard[b]	bovine stomach
lysine	104.8	115.1	127.6
histidine	16.9	13	18.8
arginine	41.9	42.1	46.9
aspartic acid	121.8	109.8	139.8
threonine[c]	67.2	68.5	98.2
serine[c]	89.7	86.4	102.8
glutamic acid	155.0	155.2	169.6
proline	62.1	58.0	108.6
glycine	75.2	64.2	100.3
alanine	81.5	85.1	98.0
cysteine	22.0	27.9	22.8
valine	76.3	82.6	77.2
methionine	21.6	21.7	17.1
isoleucine	55.5	51.6	54.6
leucine	71.4	66.9	99.0
tyrosine[c]	30.9	24.7	27.3
phenylalanine	36.2	32.1	38.7
tryptophan	ND[d]	14.6	ND

[a] Values are expressed as residues per mole. [b] From Adelstein & Klee (1981). [c] Values obtained after extrapolation to zero-time hydrolysis. [d] ND = not determined.

Sequential Phosphorylation of Myosin with Stomach and Gizzard MLCKs. For determination of whether or not bovine stomach MLCK phosphorylated the same site on smooth muscle myosin as turkey gizzard MLCK, myosin was treated initially with the turkey gizzard enzyme (Figure 4A). The time course of phosphorylation was followed and additional gizzard enzyme was added at 21 min to ensure maximal phosphorylation by this kinase. At 42 min the bovine stomach

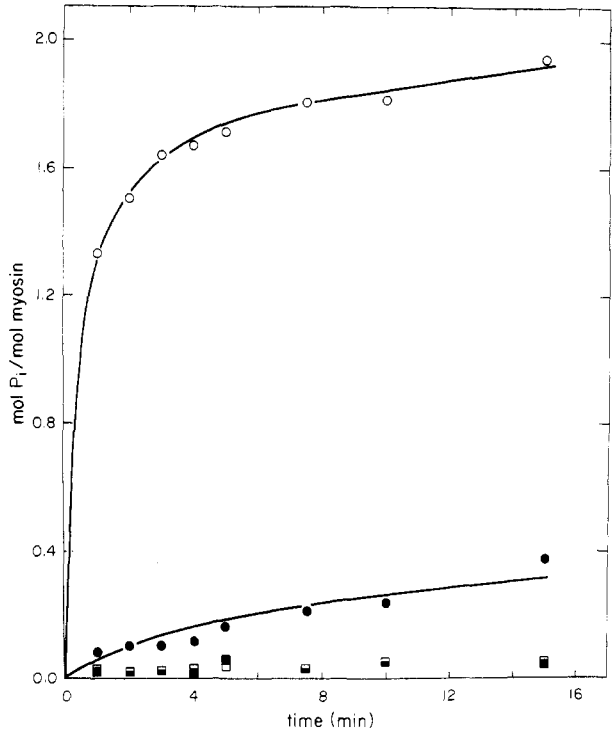

FIGURE 3: Ca^{2+} and calmodulin dependence of bovine stomach MLCK. Bovine stomach MLCK (25.8 nM) was incubated at 25 °C in 25 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 60 mM KCl, 0.75 mM [γ-^{32}P]ATP (2500 cpm/nmol), and 0.5 mg/mL turkey gizzard myosin, with (○, □) or without (●, ■) 15 μg/mL calmodulin, in the presence of 0.1 mM $CaCl_2$ (○, ●) or 1 mM EGTA (□, ■) in a total reaction volume of 4 mL. Aliquots (0.48 mL) of reaction mixtures were withdrawn at the indicated times and quenched by addition to 25% Cl_3CCOOH and 2% $NaPP_i$ (0.5 mL). Protein-bound ^{32}P was quantitated as described under Materials and Methods.

MLCK was added. If the two kinases phosphorylated different sites on the myosin molecule, one would predict an increase in phosphate incorporation following addition of the stomach enzyme. On the other hand, if the two kinases phosphorylated the same site, addition of the stomach kinase would not result in increased phosphate incorporation. It is clear from Figure 4A that the latter situation is the case. Identical observations were made in the reverse experiment; i.e., maximal phosphorylation was achieved with the stomach enzyme, and subsequent addition of gizzard MLCK resulted in no further phosphate incorporation (Figure 4B). It appears, therefore, that bovine stomach and turkey gizzard MLCKs catalyze phosphorylation of the same site on turkey gizzard myosin. Since the serine residue which was phosphorylated was not identified, it is a remote possibility that the exclusive phosphorylation at one site could also be generated by two interacting adjacent sites. The fact that bovine stomach MLCK specifically phosphorylates the 20 000-dalton light chain of gizzard myosin was demonstrated by gel electrophoresis and autoradiography (data not shown).

Phosphorylation by the Catalytic Subunit of cAMP-Dependent Protein Kinase. Bovine stomach MLCK, like its turkey gizzard counterpart, is a substrate of cAMP-dependent protein kinase (Adelstein et al., 1978; Conti & Adelstein, 1981). The rates of phosphorylation of bovine stomach and turkey gizzard MLCKs by the catalytic subunit of cAMP-dependent protein kinase are compared in Figure 5. The bovine stomach enzyme in the absence of Ca^{2+} is phosphorylated to the extent of ~2 mol/mol. In the presence of bound calmodulin, the rate of phosphorylation is considerably slower [0.014 mol of P_i (mol of kinase)$^{-1}$ min^{-1} compared with 0.081

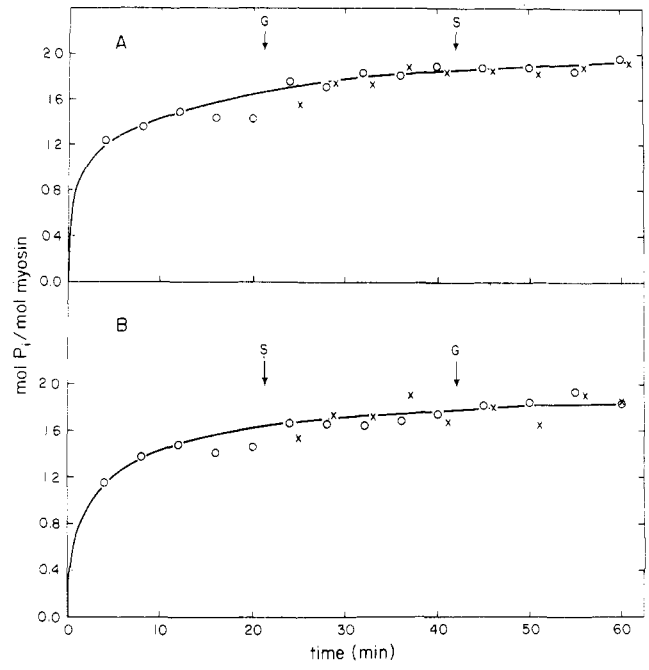

FIGURE 4: Sequential phosphorylation of myosin by bovine stomach and turkey gizzard MLCKs. (A) Turkey gizzard MLCK (20 nM) was incubated at 25 °C in 25 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 60 mM KCl, 0.1 mM $CaCl_2$, 0.75 mM [γ-^{32}P]ATP (3300 cpm/nmol), 15 μg/mL calmodulin, and 0.5 mg/mL turkey gizzard myosin. Aliquots (0.45 mL) were withdrawn at the indicated times for quantiation of protein-bound ^{32}P. The reaction mixture was divided into two equal aliquots, and additional turkey gizzard MLCK (20 nM final concentration) was added to one sample (×) at 21 min to ensure maximal myosin phosphorylation. The control sample (○) received no addition. At 42 min, bovine stomach MLCK was added to the test sample (×) to a final concentration of 25.8 nM. The control sample (○) again received no addition. (B) Conditions were exactly as in (A) except that bovine stomach MLCK (25.8 nM final concentration) was added at time 0 (○, ×) and 21 min (×) and turkey gizzard MLCK [20 nM final concentration (×)] at 42 min.

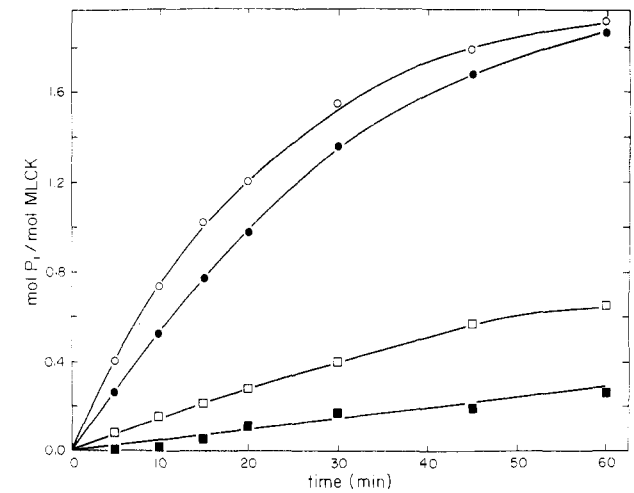

FIGURE 5: Phosphorylation of bovine stomach and turkey gizzard MLCKs by the catalytic subunit of cAMP-dependent protein kinase. Bovine stomach MLCK (0.19 mg/mL) (○, □) or turkey gizzard MLCK (0.19 mg/mL) (●, ■) was incubated at 25 °C in 15 mM Tris-HCl (pH 7.5), 1 mM DTT, 4 mM $MgCl_2$, and 1 mM [γ-^{32}P]ATP (2600 cpm/nmol) with the purified catalytic subunit of bovine cardiac cAMP-dependent protein kinase (at a weight ratio to MLCK of 1:100) and 0.1 mg/mL calmodulin in the presence of either 0.1 mM $CaCl_2$ (□, ■) or 2 mM EGTA (○, ●) in a total reaction volume of 3.5 mL. Aliquots (0.48 mL) of reaction mixtures were withdrawn at the indicated times for quantitation of protein-bound ^{32}P as described under Materials and Methods. Controls from which the protein kinase catalytic subunit was omitted exhibited no significant ^{32}P incorporation throughout the course of the 60-min incubation.

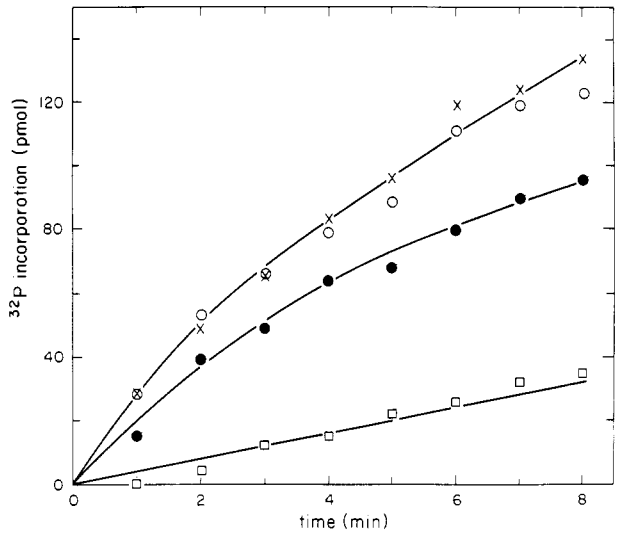

FIGURE 6: Effect of phosphorylation of bovine stomach MLCK by the catalytic subunit of cAMP-dependent protein kinase. Bovine stomach MLCK (83.3 μg/mL) was incubated at 25 °C for 90 min in 15 mM Tris-HCl (pH 7.5), 1 mM DTT, 4 mM $MgCl_2$, 2 mM EGTA, and 1 mM ATP in the presence and absence of the purified catalytic subunit of cAMP-dependent protein kinase (at a weight ratio to MLCK of 1:100) in a total reaction volume of 0.1 mL. Duplicate reaction mixtures were incubated simultaneously with 1 mM [γ-^{32}P]ATP (8500 cpm/nmol) in place of cold ATP for quantitation of the extent of phosphorylation. Aliquots (5 μL) of the reaction mixtures were then immediately assayed for MLCK activity [(○, □) phosphorylated MLCK; (×, ●) nonphosphorylated control], in the presence of 25 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 60 mM KCl, 0.1 mM $CaCl_2$, 0.5 mg/mL turkey gizzard myosin, 0.75 mM [γ-^{32}P]ATP (2500 cpm/nmol), and either a limiting (5 nM) (●, □) or saturating (0.91 μM) (×, ○) concentration of calmodulin in a total volume of 4 mL. Aliquots (0.48 mL) were withdrawn at the indicated times for quantitation of protein-bound ^{32}P as described under Materials and Methods.

mol of P_i (mol of kinase)$^{-1}$ min^{-1}] and approaches 1 mol/mol (0.65 mol/mol after 60-min incubation). These data are qualitatively similar to those obtained with the gizzard enzyme (Conti & Adelstein, 1981); however the rates of phosphorylation of the gizzard enzyme in the presence [0.005 mol of P_i (mol of kinase)$^{-1}$ min^{-1}] and absence [0.053 mol of P_i (mol of kinase)$^{-1}$ min^{-1}] of bound calmodulin are slower than the corresponding rates with the bovine stomach enzyme as substrate (Figure 5). The difference in the rates of phosphorylation for the two MLCKs was a consistent observation and was obtained with three preparations of each kinase. When the turkey gizzard enzyme was incubated with a higher ratio of protein kinase catalytic subunit (1:10 w/w), the MLCK was phosphorylated much more rapidly [initial rate ≥0.25 mol of P_i (mol of kinase)$^{-1}$ min^{-1} in the absence of Ca^{2+}–calmodulin and 0.076 mol of P_i (mol of kinase)$^{-1}$ min^{-1} in the presence of Ca^{2+}–calmodulin] to final levels of 1.8 mol/mol and 1.0 mol/mol in the absence and presence of bound calmodulin, respectively.

The effect of phosphorylation on the stomach MLCK is shown in Figure 6. In the presence of a limiting amount of calmodulin (5 nM) the rate of myosin phosphorylation catalyzed by the enzyme phosphorylated to the extent of 1.8 mol/mol was significantly less than with the control (nonphosphorylated) kinase. This inhibition could be overcome by excess calmodulin so that in the presence of saturating amounts of calmodulin (0.91 μM) the rates of myosin phosphorylation catalyzed by phosphorylated and control stomach MLCKs were indistinguishable. The data in Figure 6 show that the inhibitory effect of phosphorylation of stomach MLCK by the catalytic subunit of cAMP-dependent protein kinase is due to a decreased affinity of the kinase for calmodulin.

Discussion

The presence of MLCK in cow stomach was originally demonstrated by Frearson et al. (1976), and it was subsequently shown by Small & Sobieszek (1977) that the actin-activated myosin ATPase of pig stomach is regulated by Ca^{2+}-dependent phosphorylation–dephosphorylation of the 20 000-dalton light chain of myosin. In support of these findings, Chacko (1981) found that actomyosin of bovine stomach (the abomasal region) contained MLCK and myosin phosphatase and that there was a correlation between actin-activated myosin ATPase activity and phosphorylation of the 20 000-dalton light chain of myosin.

We report here a relatively rapid and simple procedure for the isolation of smooth muscle MLCK, which has been applied to bovine stomach and turkey gizzard (Walsh et al., 1982a). Most of the properties of the two enzymes are similar with the exception of the molecular weights. The molecular weight of the bovine stomach MLCK catalytic subunit was determined to be 155 000 by $NaDodSO_4$–polyacrylamide gradient gel electrophoresis. The fact that the stomach enzyme is indeed heavier than the 130 000-dalton gizzard kinase was firmly established by coelectrophoresis of the two purified enzymes. It is clear from the work from several laboratories that MLCKs are highly susceptible to proteolysis during purification. The platelet enzyme, for example, was originally described as a Ca^{2+}-independent kinase of M_r 78 000 (Daniel & Adelstein, 1976). It was shown later (Hathaway & Adelstein, 1979) that this represented a proteolytic fragment of a Ca^{2+}- and calmodulin-dependent MLCK of M_r 105 000. Similarly, Bremel & Shaw (1978) demonstrated Ca^{2+}-insensitive MLCK activity in mammary glands; addition of Ca^{2+}- and calmodulin-dependent gizzard MLCK to mammary actomyosin resulted in loss of Ca^{2+} sensitivity of the kinase within seconds, suggesting proteolytic degradation. Walsh et al. (1982b) showed recently that limited digestion of turkey gizzard Ca^{2+}- and calmodulin-dependent MLCK with α-chymotrypsin generates a fragment of M_r 80 000 which retains full enzymatic activity but is no longer dependent on Ca^{2+} and calmodulin for activity. These and other observations raise the possibility that MLCKs from all tissues may in fact be similar. It was suggested (Walsh et al., 1980) that MLCKs from diverse mammalain tissues share the following properties: (1) association with the contractile apparatus; (2) M_r >100 000; (3) absolute requirement for Ca^{2+} and calmodulin; and (4) phosphorylation by cAMP-dependent protein kinase. The purification of bovine stomach MLCK to homogeneity and its characterization lend support to this hypothesis. It is of further interest that the highest molecular weight previously reported for MLCK was 155 000 for the enzyme from canine skeletal and cardiac myofibrils (Walsh & Guilleux, 1981).

Our bias is that the 155 000-dalton kinase is a precursor of the lower molecular weight forms. This is supported by similarities of many properties and also by the observations that in turkey gizzard the conversion of higher molecular weight forms (M_r ~141 000 and ~136 000) to the 130 000-dalton kinase can be followed (M. P. Walsh, S. Hinkins, and D. J. Hartshorne, unpublished observations). A decrease in molecular weight as a result of proteolysis would probably be achieved by the removal of a peptide(s) from the C-terminal end of the 155 000-dalton kinase, since the N terminus of the 125 000-dalton kinase is blocked (Adelstein & Klee, 1981). The precursor–product relationship for the various kinases, however, has not been proven, and it is possible that distinct

native forms of the enzyme exist.

It is interesting and relevant to this discussion that another calmodulin-dependent enzyme, cyclic nucleotide phosphodiesterase, is also highly susceptible to proteolysis (Tucker et al., 1981). The unproteolyzed enzyme has a subunit M_r 63 000 and is essentially inactive in the absence of Ca^{2+} and calmodulin. Limited proteolysis by trypsin or an endogenous protease (with loss of $\sim$ 120 residues) yields a fragment which exhibits full enzymatic activity in the absence of Ca^{2+}–calmodulin. Furthermore, the $(Ca^{2+} + Mg^{2+})$-ATPase of erythrocyte membranes can be activated by limited proteolysis: the trypsinized enzyme exhibited the same high V_{max} and high affinity for Ca^{2+} as the native enzyme in the presence of calmodulin (Niggli et al., 1981). On the basis of limited proteolysis of MLCK, cyclic nucleotide phosphodiesterase, and $(Ca^{2+} + Mg^{2+})$-ATPase, it is likely that these, and possibly other, calmodulin-dependent enzymes share a common calmodulin-binding domain located near one terminus of the molecule. In the absence of Ca^{2+}, this domain masks the active site inhibiting enzymatic activity. Binding of calmodulin induces a conformational change relieving this inhibition. The effect of calmodulin can be mimicked by proteolytic removal of the calmodulin-binding domain.

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