Limited Proteolysis of Smooth Muscle Myosin Light Chain Kinase†

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ABSTRACT: Myosin light chain kinase plays a central role in the regulation of smooth muscle contraction. The activity of this enzyme is controlled by protein–protein interaction (the Ca^{2+}-dependent binding of calmodulin) and by phosphorylation catalyzed by cAMP-dependent protein kinase. The effects of these two regulatory mechanisms on the conformation of myosin light chain kinase and the locations of the phosphorylation sites, the calmodulin-binding site, and the active site have been probed by limited proteolysis. Phosphorylated and nonphosphorylated myosin light chain kinases were subjected to limited digestion by four proteases having different peptide bond specificities (trypsin, chymotrypsin, *Staphylococcus aureus* V8 protease, and thrombin), both in the presence and in the absence of bound calmodulin. The digests were compared in terms of gel electrophoretic pattern, distribution of phosphorylation sites, and Ca^{2+} dependence of kinase activity. A 24 500-dalton chymotryptic peptide containing both sites of phosphorylation was purified and tentatively identified as the amino-terminal peptide. The following conclusions can be drawn: (1) neither phosphorylation nor calmodulin binding induces dramatic changes in the conformation of the kinase; (2) the kinase contains two regions that are particularly susceptible to proteolytic cleavage, one located ~25 000 daltons from the amino terminus and the other near the center of the molecule; (3) the two phosphorylation sites are located within 24 500 (probably 17 500) daltons of the amino terminus; (4) the active site is located close to the center of the molecule; (5) the calmodulin-binding site is located in the amino-terminal half of the molecule, between the sites of phosphorylation and the active site, and this region is very susceptible to cleavage by trypsin.

The contractile state of smooth muscle is controlled primarily by Ca^{2+} ions via the reversible phosphorylation of myosin (Adelstein & Eisenberg, 1980; Walsh & Hartshorne, 1982). In the resting muscle, the sarcoplasmic Ca^{2+} ion concentration is low (10^{-7}–10^{-8} M), and myosin is in the nonphosphorylated state. Stimulation of the muscle cell leading to an increase in $[Ca^{2+}]$ to $\sim 5 \times 10^{-6}$ M leads to saturation of calmodulin with Ca^{2+} (4 Ca^{2+} bound/mol of calmodulin) accompanied by a significant conformational change [for recent reviews, see Cheung (1980), Klee & Vanaman (1982), and Walsh & Hartshorne (1983)]. In its altered conformation, calmodulin binds with high affinity ($K_d \sim 1$ nM) to myosin light chain kinase (MLCK)[1] (Adelstein & Klee, 1981), converting it from an inactive to an active state. The activated kinase catalyzes the phosphorylation of serine-19 on each of the two 20 000-dalton light chains of smooth muscle myosin, and the muscle contracts.

Catecholamines are known to induce relaxation in a number of smooth muscle types (Bolton, 1979), presumably via cAMP and the cAMP-dependent protein kinase. Investigations into the molecular mechanism whereby this relaxation is induced have centered on cAMP-dependent phosphorylation of membrane-associated proteins that may be involved in Ca^{2+} transport processes and on cAMP-dependent phosphorylation of contractile proteins. Adelstein et al. (1978) originally demonstrated that turkey gizzard MLCK is a substrate in vitro of the isolated catalytic subunit of cAMP-dependent protein kinase. More detailed analysis revealed that the apoenzyme of MLCK was phosphorylated at two specific sites and this chemical modification resulted in a decrease (~20-fold) in the affinity of the kinase for calmodulin (Conti & Adelstein, 1981). On the other hand, the active kinase ($4Ca^{2+}$–calmodulin–MLCK) was phosphorylated at a single site without effect. Similar observations have been made with MLCK from platelets (Hathaway et al., 1981), bovine stomach (Walsh et al., 1982a), porcine myometrium (Higashi et al., 1983), and tracheal smooth muscle (Miller et al., 1983; Nishikawa et al., 1984). These observations suggested that cAMP-dependent phosphorylation of MLCK with reduced affinity for calmodulin may result in inactivation of the kinase under physiological conditions and thereby explain the catecholamine-induced relaxation of smooth muscle. Experiments with crude biochemical systems (Silver & DiSalvo, 1979; Silver et al., 1981; Mrwa et al., 1979), intact smooth muscle (deLanerolle et al., 1984), and skinned smooth muscle fibers (Kerrick & Hoar, 1981; Rüegg et al., 1981) have provided evidence in support of this mechanism. This mechanism remains controversial, however, since significant contradictory evidence has also been obtained (Miller et al., 1983).

We describe here the results of limited proteolysis of smooth muscle MLCK with four proteases of different site specificity as a method of probing the conformational changes induced in MLCK by calmodulin binding on the one hand and by cAMP-dependent phosphorylation on the other and as a means of studying the functional domains of the kinase. In addition, a 24 500-dalton chymotryptic peptide of MLCK that contains both sites of phosphorylation has been isolated and tentatively identified as the amino-terminal peptide of the kinase.

Materials and Methods

[γ-^{32}P]ATP (10–40 and 3000 Ci/mmol) was purchased from Amersham Corp. (Oakville, Ontario, Canada), chymotrypsin A_4 and thrombin were from Boehringer Mannheim (Dorval, Quebec, Canada), TPCK–trypsin was from Worth-

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[1] Abbreviations: CaM, calmodulin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HPLC, high-pressure liquid chromatography; MLCK, myosin light chain kinase; $NaDodSO_4$, sodium dodecyl sulfate; PTH, phenylthiohydantoin; TPCK, N^{α}-tosylphenylalanine chloromethyl ketone; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

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ington (Freehold, NJ), and *Staphylococcus aureus* V8 protease was from Pierce Chemical Co. (Rockford, IL). The following molecular weight marker proteins were purchased from Sigma Chemical Co. (St. Louis, MO): myosin heavy chain (M_r 205 000), β-galactosidase (M_r 116 000), phosphorylase *b* (M_r 97 400), bovine serum albumin (M_r 66 000), ovalbumin (M_r 45 000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36 000), carbonic anhydrase (M_r 29 000), trypsinogen (M_r 24 000), trypsin inhibitor (M_r 20 100), and α-lactalbumin (M_r 14 200). Electrophoresis reagents were purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). General laboratory reagents used were analytical grade or better and were purchased from Fisher Scientific Ltd.

Calmodulin was purified from frozen bovine brains by a modification of the procedure of Gopalakrishna & Anderson (1982) as described in detail by Walsh et al. (1984). Myosin (Perechini & Hartshorne, 1981) and M_r 136 000 myosin light chain kinase (Ngai et al., 1984) were purified from chicken gizzards as previously described. The catalytic subunit of bovine cardiac type II cAMP-dependent protein kinase was purified as described by Demaille et al. (1977).

Protein concentrations were determined by the Coomassie blue dye-binding assay (Spector, 1978), using γ-globulin as the standard and dye reagent purchased from Pierce Chemical Co. (Rockford, IL), or by spectrophotometric measurements in the cases of calmodulin [$E^{1\%}_{277nm}$ = 1.9 (Klee, 1977)] and myosin [$E^{1\%}_{280nm}$ = 5.6 (Greene et al., 1983)].

Electrophoresis was performed in 7.5–20% polyacrylamide gradient slab gels (1.5 mm thick) with a 5% acrylamide stacking gel in the presence of 0.1% (w/v) $NaDodSO_4$ at 36 mA by using the discontinuous buffer system of Laemmli (1970). Gels were stained in 45% (v/v) ethanol and 10% (v/v) acetic acid containing 0.14% (w/v) Coomassie Brilliant Blue R-250 and destained in 10% (v/v) acetic acid. Stained and destained gels were dried in a Pharmacia GSD-4 slab gel drier and autoradiographed by using Kodak MinR film in a Kodak MinR cassette equipped with an intensifying screen.

Amino Acid Analysis. Triplicate samples of the purified 24 500-dalton chymotryptic peptide of MLCK (0.5 nmol each) were hydrolyzed in vacuo at 110 °C in 6 N HCl containing 0.1% (w/v) phenol and 0.02% (v/v) 2-mercaptoethanol for 24, 48, and 72 h before amino acid composition analysis in a Beckman Model 121M amino acid analyzer. Tryptophan was determined after methanesulfonic acid hydrolysis as described by Simpson et al. (1976), and cysteine was determined after performic acid oxidation as described by Hirs (1967). Amino-terminal sequence analysis was performed in a Beckman 890C sequencer equipped with a Sequemat autoconverter. The resultant PTH-amino acids were identified by HPLC using a Beckman Ultrasphere-ODS reverse-phase C-18 column.

Kinase Assays. MLCK activities were measured by quantification of [^{32}P]phosphate incorporation into isolated smooth muscle myosin as described previously (Walsh et al., 1983a). Details of reaction conditions for individual experiments are given in the text.

Phosphorylation of Myosin Light Chain Kinase. MLCK (0.25 mg/mL) was incubated at 25 °C for 30 min in 20 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 1.8 mM EGTA, and 0.35 mM [γ-^{32}P]ATP (~75 000 cpm/nmol) in the presence and absence of the isolated catalytic subunit of cAMP-dependent protein kinase (2.5 μg/mL) in a reaction volume of 2.0 mL. Reaction mixtures were placed on ice and immediately subjected to proteolysis as described below. The degree of phosphorylation obtained in the presence of the catalytic subunit was 2.0 mol of P_i/mol of MLCK; no phosphorylation was observed in the absence of the catalytic subunit.

Limited Proteolysis of Myosin Light Chain Kinase. Phosphorylated MLCK and nonphosphorylated control MLCK reaction mixtures on ice (see above) were divided into four aliquots (0.5 mL each). Calmodulin (0.1 mg/mL final concentration) was added to each sample, and $CaCl_2$ (2 mM final concentration, i.e., 0.2 mM excess over EGTA) was added to two of the phosphorylated and two of the nonphosphorylated MLCK samples. Protease was added to one of each $+Ca^{2+}$ pair and to one of each $-Ca^{2+}$ pair and digestion allowed to continue at 25 °C. The other member of each pair was incubated simultaneously without added protease and served as an undigested control. The final concentration of MLCK was 0.23 mg/mL, and reaction volume was 0.55 mL. The following protease concentrations were employed: TPCK–trypsin, 0.46 μg/mL; α-chymotrypsin, 0.46 μg/mL; *S. aureus* V8 protease, 2.3 μg/mL; thrombin, 46 μg/mL. Aliquots (80 μL) of reaction mixtures were withdrawn at t = 10, 20, 30, 40, 50, and 60 min, added to an equal volume (80 μL) of $NaDodSO_4$ gel sample buffer [50 mM Tris-HCl, pH 6.8, 1% (w/v) $NaDodSO_4$, 30% (v/v) glycerol, 0.01% (w/v) bromophenol blue, 1% (v/v) 2-mercaptoethanol], and immersed in a boiling water bath for 2 min. Aliquots (40 μL) containing 5 μg of protein were applied to gel lanes. The molecular weight marker proteins listed above were electrophoresed on each slab gel. Molecular weights of MLCK fragments were determined according to Lambin (1978). To determine the Ca^{2+} sensitivities of the MLCK fragments, digests were conducted as described above at 25 °C for 60 min and immediately assayed for MLCK activity as described above and in detail in the text. An additional chymotryptic digestion was carried out as described above but with 5 times the amount of chymotrypsin [protease:substrate = 1:100 (w/w)] prior to gel electrophoresis and examination of the Ca^{2+} sensitivity of MLCK activity in the digest.

RESULTS

Chicken gizzard MLCK, either unphosphorylated or phosphorylated to 2.0 mol of P_i/mol of kinase, was digested in the presence and absence of bound calmodulin with four different proteases: trypsin, chymotrypsin, *S. aureus* V8 protease, and thrombin. The following parameters were investigated for each digest: gel electrophoretic pattern, distribution of [^{32}P]phosphate label, and Ca^{2+} dependence of the residual MLCK activity.

Trypsin Digestion. Comparison of the time courses of tryptic digestion patterns by gel electrophoresis and autoradiography in Figure 1 reveals very little effect of either calmodulin binding or phosphorylation by the catalytic subunit of cAMP-dependent protein kinase. Digestion of the free unphosphorylated MLCK yielded two major peptides (61K and 24K); the lower molecular weight bands (17K and 16.5K) appear to be derived from calmodulin as shown in independent experiments involving digestion of calmodulin alone under identical conditions (data not shown). In the presence of bound calmodulin the unphosphorylated MLCK underwent very similar digestion to 61K and 24K peptides, but the 61K fragment was further cleaved to a 58K peptide. There was no evidence of significant degradation of calmodulin, which in the Ca^{2+}-bound form is known to be much more resistant to proteolysis than in the Ca^{2+}-free form (see Discussion). Phosphorylation of MLCK altered the digestion pattern slightly. The major 61K peptide was again generated both in the presence and in the absence of bound calmodulin. A different fragment of 25K was also generated. Again, the low

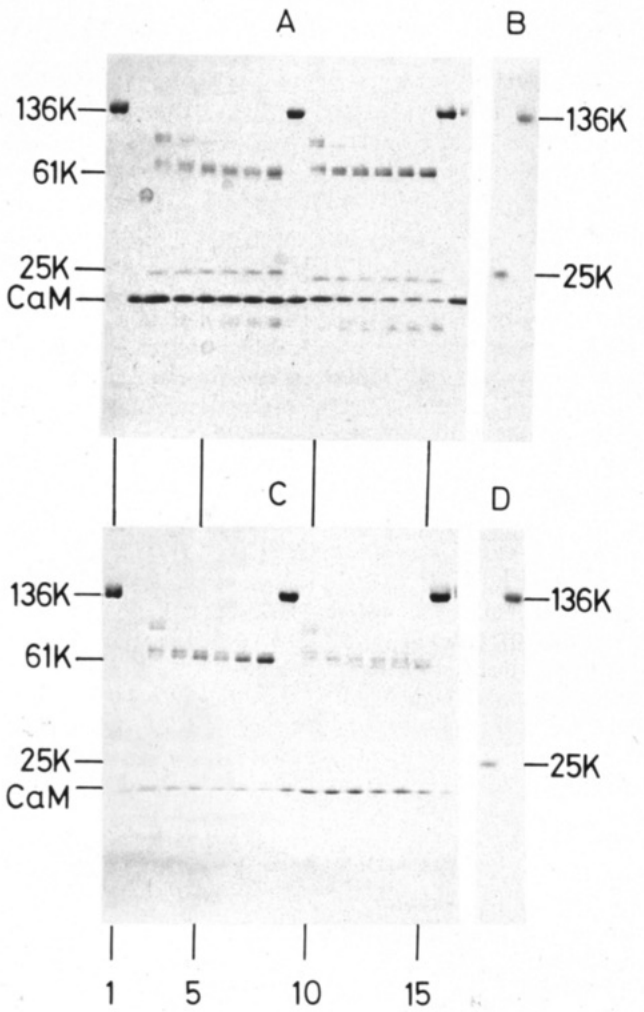

FIGURE 1: Limited proteolysis of MLCK with trypsin. MLCK (136K) was phosphorylated and digested with TPCK–trypsin in the presence and absence of bound calmodulin (CaM) as described under Materials and Methods. Digestion patterns were examined by $NaDodSO_4$–polyacrylamide gel electrophoresis and autoradiography. Key to lanes: 1 = untreated MLCK; 2 = calmodulin; 3–8 = P-MLCK–CaM (A) or P-MLCK (C) with trypsin at t = 10, 20, 30, 40, 50, and 60 min of digestion, respectively; 9 = P-MLCK–CaM (A) or P-MLCK (C) without trypsin at t = 60 min; 10–15 = MLCK (A) or MLCK–CaM (C) with trypsin at t = 10, 20, 30, 40, 50, and 60 min of digestion; 16 = MLCK (A) or MLCK–CaM (C) without trypsin at t = 60 min. (A and C) Stained gels; (B and D) autoradiograms corresponding to lanes 8 (left) and 9 (right) in (A) and (C), respectively. P-MLCK–CaM = phosphorylated MLCK with bound calmodulin; P-MLCK = phosphorylated MLCK without bound calmodulin; MLCK–CaM = unphosphorylated MLCK with bound calmodulin; MLCK = unphosphorylated MLCK without bound calmodulin.

molecular weight peptides (17.5K, 17K, and 16.5K) were derived from calmodulin.

It is apparent from the autoradiograms in Figure 1 that all the ^{32}P label was recovered in the 25K tryptic fragment, both in the presence and in the absence of bound calmodulin. The first fragments to appear had molecular weights of 91K and 25K, the remainder of the molecule (~20K) presumably having been rapidly digested to smaller pieces that are not fixed in the gel. The 91K fragment was then gradually degraded to fragments of 85K, 80K, 68K, 66K, 64K, and 61K (which was quite stable) and, in some cases, further to 58K. The 25K (24K) peptide was quite stable to further digestion whether phosphorylated or not.

Examination of Figure 6 reveals that digestion of free MLCK to 61K and 25K fragments with trypsin resulted in the appearance of Ca^{2+}-independent MLCK activity. This

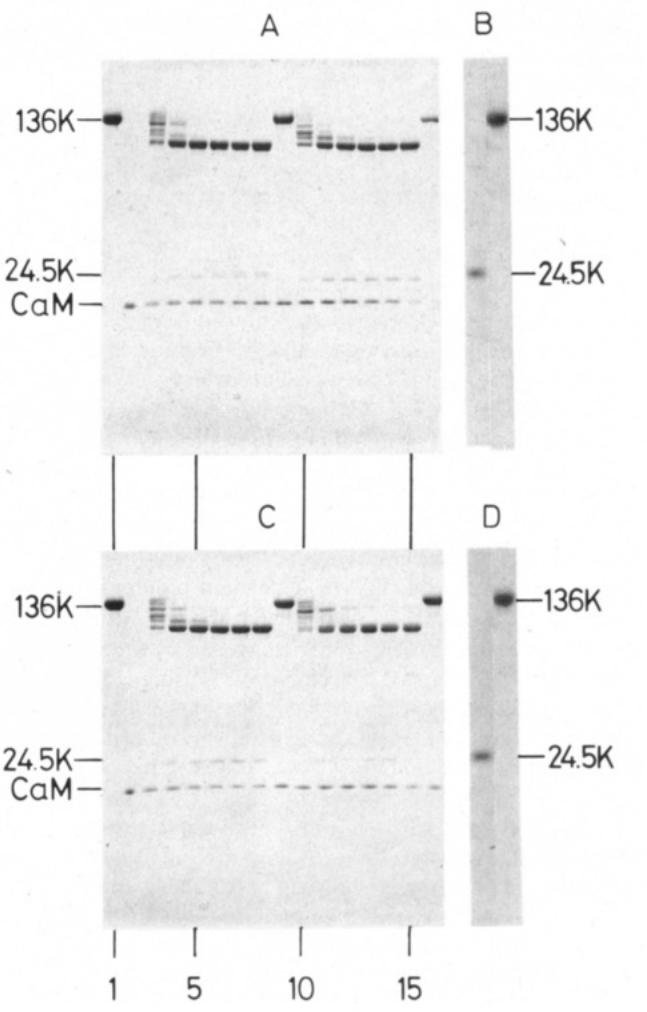

FIGURE 2: Limited proteolysis of MLCK with chymotrypsin. The arrangement is exactly as described in the legend to Figure 1 except that the protease used was chymotrypsin.

activity presumably resides in the 61K peptide, which lacks the calmodulin-binding site (see Discussion).

Chymotrypsin Digestion. The patterns of MLCK peptides obtained by chymotryptic digestion are also affected slightly by calmodulin binding or phosphorylation (Figure 2). The free unphosphorylated kinase was rapidly cleaved successively to fragments of 124K, 117K, 108K, 101K, and 95 K. The 95K fragment was quite stable to further degradation, and its formation was accompanied by the appearance of a 24K peptide. Digestion of phosphorylated MLCK generated the same 95K peptide and a 24.5K peptide that retained both phosphorylation sites. The binding of calmodulin to the phosphorylated kinase slightly altered the pattern: the 95K stable peptide was again formed, but peptides of 24.5K and 25K were also generated, the former more quickly than the latter. These peptides are not related since only the 24.5K peptide was phosphorylated and the 25K peptide appeared later than the 24.5K peptide. The 25K peptide is presumably derived from the 95K peptide, and its appearance coincides with the appearance of a 63K fragment (see below). The free phosphorylated kinase was degraded primarily to 95K and 24.5K fragments; only the 24.5K peptide was labeled.

It is apparent from the data in Figure 6A that digestion of unphosphorylated, calmodulin-free MLCK with chymotrypsin to the level of 95K + 24K peptides resulted in no loss of Ca^{2+} sensitivity of the enzyme. When the enzyme was digested further, with 5-fold more chymotrypsin, to a 63K peptide (Figure 3), Ca^{2+}-independent activity appeared (Figure 6B).

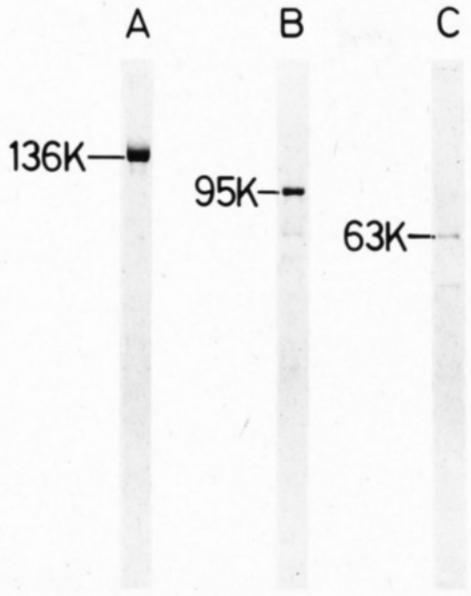

FIGURE 3: Further proteolysis of MLCK with chymotrypsin. MLCK was incubated at 25 °C for 60 min in the absence (A) or presence of chymotrypsin at a protease:substrate ratio of 1:500 (B) or 1:100 (C) as described under Materials and Methods prior to analysis by $NaDodSO_4$–polyacrylamide gradient gel electrophoresis and staining with Coomassie blue.

This activity is presumably associated with the 63K chymotryptic fragment, which therefore resembles the 61K tryptic fragment described above.

S. aureus V8 Protease Digestion. The pattern of digestion was again affected very little either by calmodulin binding or by phosphorylation, only slight differences in rate of degradation being observed (Figure 4). In each case, MLCK was degraded successively to major fragments of 112K, 93K, and 71K. These three fragments all contained [^{32}P]phosphate when phosphorylated MLCK was digested. Proteolysis was observed to occur slightly faster in the presence than in the absence of bound calmodulin, and a 58K peptide began to appear. The 71K peptide apparently contained both phosphorylation sites, and prolonged digestion of the calmodulin-bound form of the enzyme resulted in appearance of a labeled 17.5K peptide.

Proteolysis of MLCK to the level of the 71K peptide had no effect on the activity or Ca^{2+} dependence of the enzyme (Figure 6A), suggesting that the 71K fragment retains full enzymatic activity and the calmodulin-binding site. This peptide also retained both phosphorylation sites.

Thrombin Digestion. MLCK was highly resistant to proteolysis by thrombin. Even at a protease:substrate ratio of 1:5 (w/w), relatively little proteolysis of the kinase was observed (Figure 5). The proteolytic patterns were again little affected by calmodulin binding or phosphorylation. The free unphosphorylated MLCK was most rapidly proteolyzed, generating major fragments of 108K and 24.5K (Figure 5, lane 2). Phosphorylation slowed the rate of proteolysis, yielding only a small amount of the 24.5K peptide, which was phosphorylated (Figure 5, lane 3). The presence of bound calmodulin slowed the rate of proteolysis further, and the 24.5K peptide was not observed (Figure 5, lanes 1 and 4).

Purification of the 24 500-Dalton Chymotryptic Phosphopeptide of MLCK. MLCK was phosphorylated as described under Materials and Methods and digested with chymotrypsin [1:500 (w/w) protease:substrate] for 60 min at 25 °C. The digest (Figure 7, lane 2) was immersed in a boiling water bath, heated to 80–85 °C and maintained at this temperature for

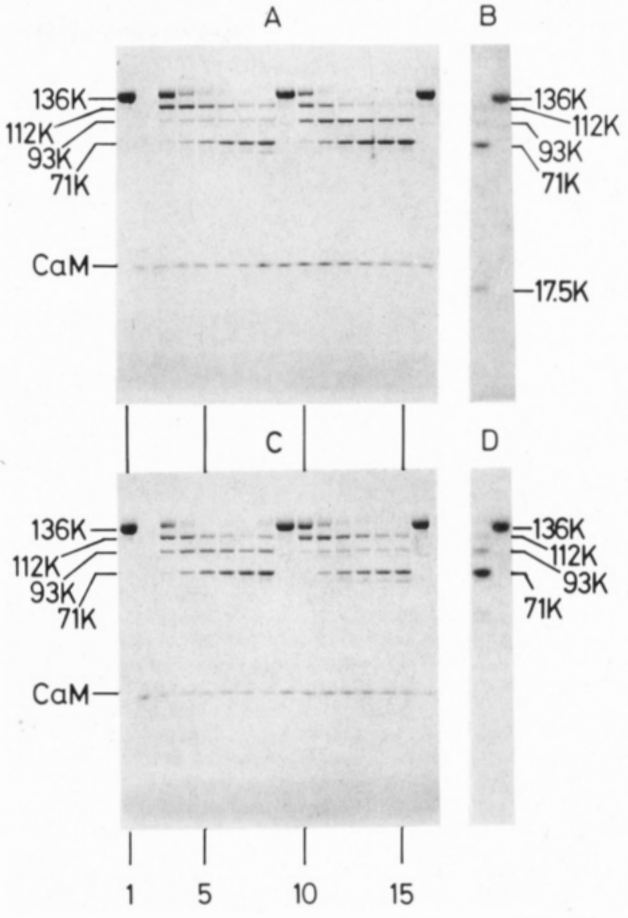

FIGURE 4: Limited proteolysis of MLCK with *S. aureus* V8 protease. The arrangement is exactly as described in the legend to Figure 1 except that the protease used was the *S. aureus* V8 protease.

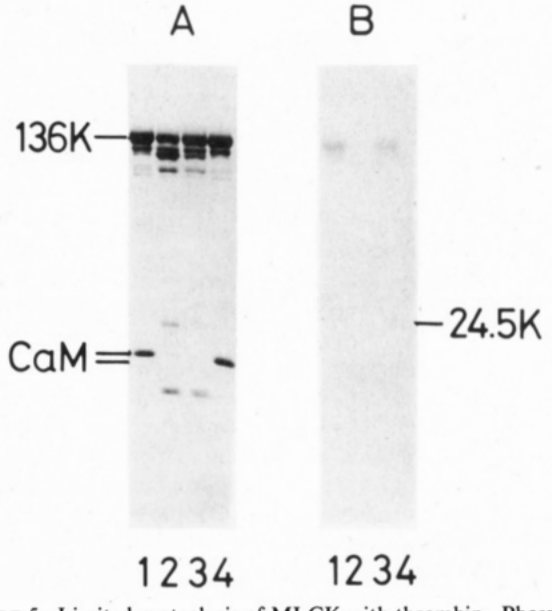

FIGURE 5: Limited proteolysis of MLCK with thrombin. Phosphorylated MLCK and unphosphorylated MLCK were incubated with thrombin in the presence and absence of bound calmodulin at 25 °C for 60 min, as described under Materials and Methods, prior to gel electrophoresis (A) and autoradiography (B). Key to lanes: 1 = P-MLCK–CaM; 2 = MLCK; 3 = P-MLCK; 4 = MLCK–CaM. For identification of abbreviations, see legend to Figure 1.

2 min, cooled on ice, and centrifuged at 37000*g* for 15 min to remove denatured proteins. The supernatant contained exclusively the 24 500-dalton phosphopeptide (Figure 7, lane 4), which was labeled with [^{32}P]phosphate (Figure 7, lane 5).

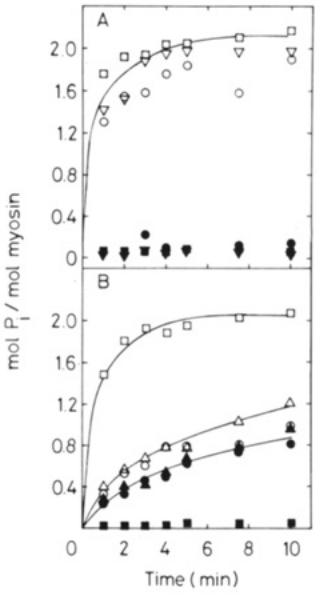

FIGURE 6: Ca^{2+} sensitivities of proteolytic digests of MLCK. MLCK (0.25 mg/mL) was incubated at 25 °C for 60 min in 20 mM Tris-HCl (pH 7.5) and 1 mM EGTA either by itself (□ and ■ in panels A and B) or in the presence of trypsin (0.5 μg/mL; △ and ▲ in panel B), chymotrypsin (0.5 μg/mL in panel A or 2.5 μg/mL in panel B; ○ and ●), or *S. aureus* V8 protease (2.5 μg/mL in panel A; ▽ and ▼). Reactions were quenched by freezing in dry ice, and MLCK activity was assayed immediately as follows: The digested MLCKs (5 μg/mL) or undigested controls (5 μg/mL) were incubated at 25 °C in 25 mM Tris-HCl (pH 7.5), 60 mM KCl, 30 μg/mL calmodulin, 0.75 mM [γ-^{32}P]ATP (~4000 cpm/nmol), and 0.1 mM $CaCl_2$ (open symbols) or 1 mM EGTA (closed symbols) with smooth muscle myosin (0.5 mg/mL). Reaction volumes = 4.0 mL. Phosphorylation was initiated by addition of [γ-^{32}P]ATP. Aliquots (0.5 mL) of reaction mixtures were withdrawn at the indicated times for quantification of protein-bound [^{32}P]phosphate as described under Materials and Methods.

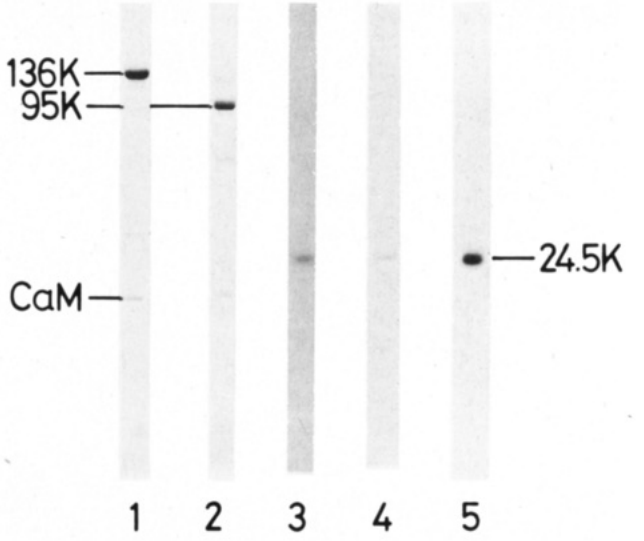

FIGURE 7: Purification of the 24 500-dalton chymotryptic phosphopeptide of MLCK. Myosin light chain kinase was phosphorylated and digested with chymotrypsin in the absence of Ca^{2+} for 60 min as described under Materials and Methods. The 24 500-dalton phosphopeptide was purified as described under Results. Key to lanes: 1 = undigested MLCK + calmodulin; 2 = chymotryptic digest of phosphorylated MLCK; 3 = autoradiogram corresponding to gel of lane 2; 4 = heat treatment supernatant of digest; 5 = autoradiogram corresponding to gel of lane 4.

Table I shows the amino acid composition of the purified phosphopeptide compared with that of the native MLCK. The distribution of amino acids in the phosphopeptide is very similar to that in the intact enzyme. Amino-terminal sequence analysis revealed that the amino terminus of the phosphopeptide, like that of intact MLCK, is blocked. Amino-terminal sequence analysis of myoglobin performed immediately before and after this analysis of the phosphopeptide successfully yielded at least the first 12 amino acids. The phosphopeptide therefore probably represents the amino-terminal region of MLCK, and both phosphorylation sites are located within 24 500 daltons of the amino terminus.

Discussion

Myosin light chain kinase plays a central role in the regulation of smooth muscle contraction and probably also nonmuscle motile processes such as exo- and endocytosis, movement of cellular organelles, and cell migration (Adelstein & Eisenberg, 1980; Walsh & Hartshorne, 1982). The activity of this enyzme is regulated by Ca^{2+} ion via the Ca^{2+}-dependent binding of calmodulin (Dabrowska et al., 1978) and by cAMP via phosphorylation of the MLCK catalyzed by cAMP-dependent protein kinase (Adelstein et al., 1978). The binding of calmodulin converts the MLCK from an inactive to an active conformation (Dabrowska et al., 1978), whereas the phosphorylation of MLCK at two specific sites reduces the affinity of the kinase for calmodulin approximately 20-fold (Conti & Adelstein, 1981) or even up to ~500-fold as estimated by fluorescence measurements (Malencik et al., 1982).

We report here the results of studies of limited proteolysis of smooth muscle MLCK as a probe of the structure–function relations of this enzyme. This approach has already proved very useful in studying the structure–function relations of calmodulin. Ca^{2+} binding to calmodulin induces rather extensive conformational changes that affect its susceptibility to proteolytic attack and the sites of cleavage. Thus, in the absence of Ca^{2+}, calmodulin is much more susceptible to tryptic cleavage than in the presence of Ca^{2+}, and the sites of cleavage under conditions of limited proteolysis are different in the absence and presence of bound Ca^{2+} (Walsh et al., 1977). The effects of four different proteases on the digestion patterns, distribution of phosphorylation sites, and Ca^{2+} sensitivity of enzymatic activity of free and phosphorylated MLCKs and their corresponding calmodulin-bound forms were examined. The proteases used exhibit different cleavage site specificities: trypsin cleaves at the carboxy-terminal side of lysine and arginine residues (Bergmann et al., 1939); chymotrypsin cleaves at the carboxy-terminal side of bulky hydrophobic residues, e.g., phenylalanine, tyrosine, tryptophan, leucine, and isoleucine (Bergmann & Fruton, 1937); *S. aureus* V8 protease cleaves at the carboxy-terminal side of aspartate and glutamate residues (Drapeau et al., 1972); and thrombin is specific for cleavage at the carboxy-terminal side of arginine residues (Blomback et al., 1977). Such an approach permits a study of the conformational changes induced in MLCK by calmodulin binding and phosphorylation that may affect regions of the molecule susceptible to proteolytic cleavage. The digestion patterns revealed by gel electrophoresis were altered only slightly by either calmodulin binding or phosphorylation. This was observed with each of the four proteases used.

Figure 8 summarizes the results of these proteolysis studies to facilitate the following discussion. The positioning of some of the peptides is tentative, as described below. Trypsin cleaved the kinase into two major fragments (61K and 24K). Phosphorylation altered this cleavage slightly to yield 61K and 25K peptides; the latter retained both sites of phosphorylation. The binding of calmodulin led to generation of 58K and 24K peptides from the unphosphorylated kinase and 61K and 25K peptides from the phosphorylated kinase. Again the 25K peptide retained both sites of phosphorylation. In the case of

Table I: Amino Acid Compositions of MLCK and 24 500-Dalton Phosphopeptide of MLCK

residue	residues/mol[a]	
	136K MLCK[b]	phosphopeptide
lysine	120.6 (9.7)	19.3 (9.4)
histidine	15.1 (1.2)	2.8 (1.4)
arginine	47.7 (3.8)	5.4 (2.6)
aspartic acid	120.6 (9.7)	20.7 (10.1)
threonine[c]	76.2 (6.1)	11.2 (5.5)
serine[c]	109.8 (8.8)	14.1 (6.9)
glutamic acid	169.2 (13.6)	32.1 (15.7)
proline	65.2 (5.2)	12.7 (6.2)
glycine	82.4 (6.6)	16.7 (8.2)
alanine	105.9 (8.5)	17.5 (8.6)
cysteine[d]	28.2 (2.3)	6.6 (3.2)
valine	76.3 (6.1)	13.2 (6.5)
methionine	26.0 (2.1)	4.0 (2.0)
isoleucine	46.2 (3.7)	6.7 (3.3)
leucine	71.7 (5.8)	9.2 (4.5)
tyrosine	29.2 (2.3)	5.0 (2.4)
phenylalanine	34.8 (2.8)	5.0 (2.4)
tryptophan[e]	20.5 (1.6)	2.1 (1.0)

[a] Values represent the means of triplicate determinations at 3 times of hydrolysis (24, 48, and 72 h). Numbers in parentheses express each residue as a percent of total residues. [b] P. K. Ngai and M. P. Walsh, submitted for publication. [c] Extrapolated to zero time of hydrolysis. [d] Determined according to Hirs (1967). [e] Determined according to Simpson et al. (1976).

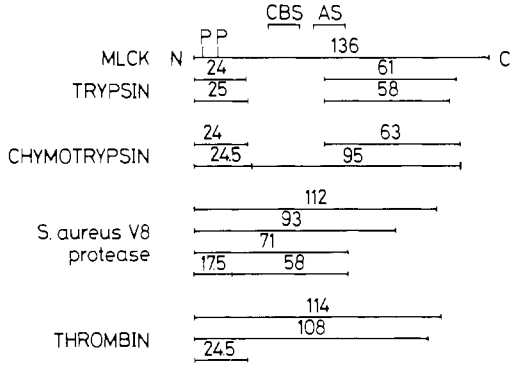

FIGURE 8: Proteolytic fragments derived from MLCK. Numbers above each peptide denote molecular weights in thousands. Sites of phosphorylation are indicated by P, the CaM-binding site is indicated by CBS, and the active site is indicated by AS. Peptide fragments are grouped according to the protease utilized and are aligned with the undigested 136K MLCK; some of these position assignments are tentative (see Discussion). The amino and carboxy termini of MLCK are denoted N and C, respectively.

chymotrypsin, two major peptides were again generated: a 95K peptide in each case and either a 24K peptide from the free kinase or a 24.5K peptide from phosphorylated MLCK, calmodulin-bound MLCK, and phosphorylated, calmodulin-bound MLCK. There appear, therefore, to be two major chymotryptic cleavage points in MLCK. One is cleaved in phosphorylated MLCK to yield a 24.5K-labeled peptide in the presence and absence of bound calmodulin. A second cleavage yields a 95K peptide in all four cases. The 24.5K-labeled peptide produced by chymotryptic digestion of phosphorylated MLCK without bound calmodulin was purified and found to have a blocked amino terminus, in common with the intact enzyme (Adelstein & Klee, 1981). This enables us to place the amino-terminal peptides on the basis of phosphorylation sites. The 95K peptide is tentatively placed adjoining the phosphopeptide in Figure 8, although this peptide could equally as likely be located at the carboxy terminus.

The *S. aureus* V8 protease cleaved MLCK successively to amino-terminal peptides of 112K, 93K, and 71K. These cleavage sites were not changed by either phosphorylation or calmodulin binding; these affected only slightly the rates of proteolysis. The 71K peptide began to undergo further digestion in the presence of bound calmodulin, apparently to 58K + 17.5K peptides. Only the latter was labeled, suggesting that the phosphorylation sites are located within 17 500 daltons of the amino terminus.

MLCK was quite resistant to proteolysis by thrombin, but the use of a high protease:substrate ratio [1:5 (w/w)] enabled partial digestion to be achieved. Major cleavage sites yielding amino-terminal peptides of 114K and 108K were observed, and in the case of calmodulin-free MLCK, an amino-terminal peptide of 24.5K was released. In the case of phosphorylated MLCK this peptide was labeled.

The following main conclusions can be drawn from these experimental results, which enable reconstruction of the peptide arrangements shown in Figure 8: (1) The two phosphorylation sites are located close to the amino terminus since they are recovered in a 24 500-dalton chymotryptic peptide that contains, like the native enzyme, a blocked amino terminus. (2) The 61K tryptic fragment and 63K chymotryptic fragment, which exhibit Ca^{2+}-independent MLCK activity, retain at least part of the active site but lack both phosphorylation sites and the calmodulin (CaM) binding site. (3) The 71K *S. aureus* V8 protease peptide retains full enzymatic activity, Ca^{2+}/CaM dependence, and both sites of phosphorylation and must therefore represent the amino-terminal half of the molecule. (4) The 95K chymotryptic fragment exhibits Ca^{2+}/CaM dependence but lacks both phosphorylation sites; it is therefore not the amino-terminal peptide. Its formation is accompanied by the appearance of a 24.5K amino-terminal peptide containing both sites of phosphorylation. (5) Cleavage of the 95K chymotryptic fragment to a 63K peptide is accompanied by loss of Ca^{2+} sensitivity and reduced specific activity of the kinase, suggesting that the CaM-binding site is removed or destroyed and the active site partially affected. On the basis of these findings, the phosphorylation sites can be placed close to the amino terminus, the CaM-binding site coming next and then the active site being located near the center of the molecule. All three domains are retained in a 71K *S. aureus* V8 protease fragment. The carboxy-terminal half of the molecule presumably contains other functionally important domains, e.g., the actin-binding domain (Dabrowska et al., 1982; Sobue et al., 1982).

We have shown previously that partially purified turkey gizzard MLCK was cleaved by chymotrypsin to yield a 78.5K fragment, which was subsequently purified (Walsh et al., 1982b). This peptide lacked the two phosphorylation sites and did not bind calmodulin. It did, however, retain full enzymatic activity that was Ca^{2+} independent. The data presented here indicate that this peptide was not generated by using the pure MLCK as substrate for chymotrypsin. At the same protease:substrate ratio [1:500 (w/w)] a 95K peptide was produced that retained Ca^{2+}-dependent enzymatic activity. More complete digestion of pure MLCK [protease:substrate = 1:100 (w/w)] generated a 63K peptide that retained MLCK activity (albeit reduced) with loss of Ca^{2+} sensitivity. Trypsin digestion produced a similar peptide of 61K that lacked both phosphorylation sites and exhibited Ca^{2+}-independent MLCK activity. The 78.5K chymotryptic fragment previously isolated may represent an intermediate peptide between the 95K and 63K peptides. Its formation may be caused by the presence of actin bound to MLCK in the impure preparation used for its generation (Walsh et al., 1982b). Ca^{2+}-independent forms of MLCK have also been generated by tryptic digestion of the chicken gizzard enzyme (Tanaka et al., 1980; Adelstein et al.,

1982; Cande et al., 1983) and the rabbit skeletal muscle enzyme (Tanaka et al., 1980; Srivastava & Hartshorne, 1983).

Limited digestion of skeletal muscle MLCK (M_r 70 300) with trypsin cleaved the molecule into two major fragments: a globular head fragment (M_r ~36 000), containing the active site and the calmodulin-binding site, and a highly asymmetric tail fragment (M_r ~33 000) (Mayr & Heilmeyer, 1983). Binding of calmodulin to the head induced an increase in asymmetry and α-helix content (Mayr & Heilmeyer, 1983). Johnson et al. (1981) observed an ~30% increase in tryptophan fluorescence upon binding of calmodulin to skeletal muscle MLCK, indicating a significant calmodulin-induced conformational change. In contrast, Malencik et al. (1982) observed only subtle changes in the fluorescence emission and polarization excitation spectra of the turkey gizzard enzyme. Our data are consistent with relatively small conformational changes occurring in the smooth muscle enzyme upon binding of calmodulin, phosphorylation by cAMP-dependent protein kinase, or both. MLCK of smooth muscle therefore appears to be quite different from the skeletal muscle enzyme. Consistent with this conclusion, comparison of the amino acid compositions of rabbit skeletal muscle MLCK and turkey gizzard MLCK suggested some similarity, although sequence homology was not indicated (Crouch et al., 1981).

Registry No. MLCK, 51845-53-5.

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