A 40-kDa fragment of chicken smooth muscle myosin light chain kinase was produced and partially purified from a bacterial expression system. This fragment exhibits calmodulin binding and substrate phosphorylation properties similar to those of the isolated chicken gizzard enzyme. A series of 3'-deletion mutants was prepared and used to produce proteins with the same NH2 terminus but with COOH termini varying over 180 amino acids. Results show that truncation of the enzyme at Ser-512 (based on the amino acid numbering system described for the partial cDNA clone by Guerriero, V., Jr., Russo, M. A., Olson, N. J., Putkey, J. A., and Means, A. R. (1986) Biochemistry 25, 8372-8381) does not alter calmodulin binding, calmodulin regulation, or enzymatic properties. Removal of an additional 5 residues from the COOH terminus completely inhibits calmodulin binding and results in an inactive kinase that can be fully activated by limited proteolysis. Site specific mutations within these 5 residues demonstrate that Gly-508 and Arg-509 are independently involved in calmodulin-dependent binding and activation of myosin light chain kinase. Truncation of the enzyme at residues within the protein kinase catalytic domain results in inactive protein that cannot be activated by proteolysis.

The vertebrate myosin light chain kinases (MLCK) are Ca2+-calmodulin-dependent enzymes involved in the regulation of contractility (1, 2). In smooth muscle phosphorylation of the 20-kDa light chain of myosin is rate limiting for contraction (2). Because of the obvious physiological relevance of smooth muscle MLCK, it is a much studied enzyme. Initial information on the primary sequence of the chicken gizzard MLCK was obtained by analysis of a cDNA clone that represented the COOH-terminal 60% of the amino acid coding region (3). The calmodulin (CaM)-binding region was defined to be encoded by a 192-nucleotide fragment of the cDNA that was positioned very close to the COOH-end of the protein kinase catalytic homology (3). Subsequent studies by Lukas et al. (4) and Kemp et al. (5) revealed that the expression of the CaM-binding region encompassed amino acids 480-510 based on the numbering system established by Guerriero et al. (3) for the partial cDNA clone. Lukas et al. (4) found that a synthetic peptide analog of amino acids 493-510 bound CaM with an affinity similar to the native enzyme. Kemp et al. (5) observed that residues 485-505 exhibited significant similarity to the myosin light chain substrate and that a synthetic peptide analog to this pseudosubstrate region served as an antagonist of light chain phosphorylation. The CaM binding and the pseudosubstrate regions of chicken gizzard MLCK overlap. The NH2-terminal two-thirds (484-504) act as a pseudosubstrate and the COOH-terminal two-thirds (489-510) encode the CaM binding portion. Because these functional sequences have been defined using synthetic peptides, they have been referred to as pseudosubstrate and CaM-binding prototopes, respectively (6).

Proteolysis of smooth muscle MLCK and many other Ca2+/CaM-dependent enzymes releases a constitutively active fragment (8-16). Ikebe et al. (17) demonstrated that the activation of chicken gizzard MLCK was a two-step process in which an inactive 64-kDa peptide was first generated by trypsinolysis. Continued proteolysis of this inactive fragment produced an active 61-kDa protein. Pearson et al. (6) revealed that the COOH-terminal residue of the inactive 64-kDa protein was Arg-505 in the CaM-binding prototope. However, the relative importance of individual residues required for CaM binding remains to be determined.

As an initial step to precisely determine the importance of specific amino acids and regions required for the functional activities of smooth muscle MLCK, we have introduced a portion of the cDNA into a bacterial expression vector. One protein produced is 40 kDa and contains the necessary information to serve as a Ca2+/CaM-dependent MLCK and terminates in Ser-512. The enzymatic properties of this fragment are similar to those of the native 130-kDa chicken gizzard enzyme. A series of 3'-deletion mutants were generated that truncate the bacterially expressed 40-kDa MLCK from the COOH-terminal end. Removal of only five amino acids results in generation of an inactive protein that does not bind CaM but can be activated by limited proteolysis. Site specific mutagenesis was used to demonstrate that Gly-508 and Arg-509 are critical amino acids for activation of MLCK by binding CaM.

EXPERIMENTAL PROCEDURES

Materials—Reagents were purchased from the following companies: nalidixic acid, Sigma; [γ-32P]ATP (6000 Ci/mmol), Amersham Corp.; 125I-protein A, ICN Radiochemicals; nitrocellulose (0.45 μm)
and electrophoresis reagents, Bio-Rad. Enzymes used in DNA manipulations were obtained from Bethesda Research Laboratories or Boehringer Mannheim and were used according to the manufacturer's specifications.

**Plasmids and Bacterial Strains**—The expression vector pOTS (18) was kindly provided by Dr. Martin Schlegel (Smith, Kline and French Laboratories). *Escherichia coli* strain AR120, a *colE* defective lysogen inducible by nalidixic acid (19), was used for expression of MLCK cDNA.

**Construction of the Expression Vector**—The plasmid MK2.1 containing the cDNA for MLCK (3) was digested with restriction enzymes NcoI and Hpal to generate a 1456-bp fragment. This fragment was blunt-ended with Klenow enzyme. The vector pOTS was linearized with *BamHI*, blunt-ended with Klenow enzyme, and ligated to the 1456-bp MLCK insert. Following transformation in bacteria, colonies were screened with the radiolabeled 1456-bp MLCK cDNA fragment to detect the plasmids containing the insert. DNA was isolated from positive colonies and digested with SacI to establish the orientation of the insert. A clone with the proper insert orientation was designated as pOTS-MK1.4.

**Preparation of Deletion Mutants by Exonuclease III Digestion**—Expression plasmid pOTS-MK1.4 was cleaved with the restriction enzyme *BamHI*. Re-ligation of this clone following *S1* nuclease digestion of the blunt-ends produced the truncated plasmid pOTS-MK1.1. pOTS-MK1.4 DNA linearized with NcoI was also incubated with Exonuclease III at 37 °C in a buffer containing 50 mM Tris- *HCl* (pH 8.0), 10 mM MgCl₂, and 100 mM NaCl (20). At different time intervals aliquots of the solution were taken, digested with *S1* nuclease at 25 °C to create blunt ends, and ligated to recircularize the plasmid. Transformation of ligated DNA into *E. coli* was carried out as described by Hanahan (21). DNA was isolated from individual colonies and then digested with *SacI* to map the extent of the deletions. The precise boundary of each deletion mutant was determined by the dideoxy DNA sequencing technique (22).

**Site-specific Mutagenesis**—Oligonucleotide-directed site-specific mutagenesis was performed using a Mutagen-Gen kit (Bio-Rad). A 1.1-kb *PstI*-SstI fragment from pOTS-MK1.4 plasmid was inserted into *PstI*-SstI *EcoRV* (Biotechnics, Inc.) vector. Using oligonucleotides as primer Gly-508 and Arg-509 were mutated to Glu and Ile, respectively. All mutations were confirmed by DNA sequence analysis. MLCK insert with the specific mutation was isolated from the vector POTS and blunt-ended with Klenow enzyme. The vector pOTS was linearized with *BamHI*, blunt-ended with Klenow enzyme, and ligated to the 1456-bp MLCK insert. Following transformation in bacteria, colonies were screened with the radiolabeled 1456-bp MLCK cDNA fragment to detect the plasmids containing the insert. DNA was isolated from positive colonies and digested with SacI to establish the orientation of the insert. A clone with the proper insert orientation was designated as pOTS-MK1.4.

**Induction and Cell Lysis**—*E. coli* strain AR20 cells were transformed with pOTS, pOTS-MK1.4, pOTS-MK1.1, site-specific mutants, Kinase (−) mutant, and all of the Exonuclease III deletion mutants. The transformed bacteria were grown in LB broth containing ampicillin (50 μg/ml) at 37 °C to late log phase. Cells were collected by centrifugation and washed with the addition of nalidixic acid to a final concentration of 40 μg/ml as described by Mott et al. (19). After a 15-h induction, bacterial cells were pelleted by centrifugation. The pellet was resuspended in 1.5 volumes of 5% sucrose, 0.5 M Tris (pH 8.0), Lysozyme (3 mg/ml) to 15% of total volume and 0.25 M EDTA (pH 7.0) to 2% total volume were then added. The suspension was shaken at 37 °C for 30 min. This was followed by addition of an equal volume of 2% Brij (ε non-ionic detergent) in 0.05 M Tris- *HCl* (pH 8.0). The mixture was again shaken at 37 °C for 20 min followed by sonication for 15 s. It was then centrifuged at 10,000 rpm for 10 min, the pellet was discarded and the supernate was assayed for MLCK activity.

**Protein Purification**—Chicken gizzard MLCK was purified according to Adelstein and Klee (23). Myosin from chicken gizzard was prepared as described by Crouch et al. (24). The light chains of myosin were extracted by the guanidine hydrochloride method of Perrie and Perry (25). Digested myosin light chains were separated from calmodulin by DEAE-Sepharose chromatography (23). Calmodulin was purified from bacterial lysates as described by Putkey et al. (26).

**Purification of MK-40K and MK-50K Proteins from Bacterial Cells**—One-liter cultures of bacteria transformed with either pOTS-MK1.1 or pOTS-MK1.4 were grown in an induction medium and induced with 0.5 M IPTG. Following induction and 3 h of incubation at 37 °C, the bacteria were centrifuged. The protein pellet was discarded and more solid ammonium sulfate was added to the supernatant to achieve 60% saturation. As before, the solution was stirred for 20 min and then centrifuged. The protein pellet was discarded and more ammonium sulfate was added to the supernate to achieve 60% saturation. As before, the solution was stirred for 20 min and centrifuged. The supernate was discarded and the pellet was dissolved in a minimal volume of buffer A (50 mM HEPES, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, and 0.1 mM EGTA). The protein solution was dialyzed for 16 h against the same buffer with two changes. The dialyzed solution was clarified by centrifugation at 10,000 × g for 15 min. The protein solution (200 mg) was then applied to a 10-ml DEAE-cellulose column pre-equilibrated with 20 mM NaCl in buffer A. The column was washed thoroughly with the same buffer and the bound proteins were eluted with an 80-ml linear gradient of 40–500 mM NaCl in buffer A. MLCK activity was assayed in each gradient fraction using myosin light chain as substrate. The active fractions were pooled, dialyzed against buffer A (50 mM HEPES, pH 7.5, 1 mM dithiothreitol, 1 mM EGTA), and dialyzed and loaded onto a 2-ml heparin-Sepharose column. Bound proteins were eluted with a 25-ml linear gradient of 50–200 mM NaCl in buffer A. The fractions containing MLCK activity were pooled and dialyzed against buffer B (50 mM HEPES, pH 7.5, 1 mM dithiothreitol, 1 mM CaCl₂) and loaded onto a 2-ml CaM-Sepharose column previously equilibrated with buffer B. Most of the protein did not bind to the column and came out in the flow-through. Bound proteins were eluted with buffer C containing 500 mM NaCl, 10 mM MgCl₂, 0.5 mM EGTA, and 1 mM dithiothreitol. Fractions were again assayed for MLCK activity. The active fractions were pooled and concentrated by loading onto a 1-ml DEAE-cellulose column. Bound proteins were eluted with buffer A containing 400 mM NaCl. The eluted fractions were assayed for MLCK activity and stored in small aliquots, and then stored frozen at −80 °C. Protein concentrations were determined at different steps of purification by the Bradford assay (27).

The bacterial extracts expressing MLCK mutants terminating at Lys-476 and Lys-445 were purified through DEAE-cellulose and heparin-Sepharose chromatography as described above. The mutant proteins were detected in the gradient fractions using immunoblot analysis.

**Assay of MLCK Activity**—MLCK activity was assayed by the filter paper procedure of Corbin and Reimann (28) either with isolated *M. ribosum* light chains (20 μM) or native myosin (12 μM) substrate. Proteins were mixed in reaction volume (0.1 ml) containing 50 mM HEPES (pH 7.5), 10 mM MgCl₂, 0.2 mM CaCl₂, 0.1 mM [γ-³²P]ATP (0.5 Ci/mmol), and 2 mM myosin light chain kinase at 25 °C. Unless otherwise indicated, 10 μM CaM was included in the reaction. The assay was initiated by addition of the kinase.

**Immunoblotting and Calmodulin Overlack Assay**—Protein samples were first electrophoresed on a 7.5–15% polyacrylamide gradient slab gel using the discontinuous buffer system of Laemmli (29). After electrophoresis proteins were transferred to nitrocellulose membranes using a Transblot electrophoresis system. For immunological detection of proteins, the nitrocellulose membrane with the bound proteins was first soaked in buffer C containing 10 mM Tris- *HCl*, pH 7.6, 0.9% NaCl, and 0.1% Tween-20 for 5 min, then rinsed 5% Blotto in buffer C. After rinsing, the resultant construct, designated POTS-MK1.4, containing a partial portion of the smooth muscle MLCK cDNA (Asp'47-Glu'108), was digested with NcoI and religated in the same way as pOTS-MK1.1. pOTS-MK1.4 DNA linearized with NcoI was also incubated with Exonuclease III at 37 °C in a buffer containing 50 mM Tris- *HCl* (pH 7.4), 10 mM MgCl₂, 0.2 mM CaCl₂, 0.1 mM [γ-³²P]ATP (0.5 Ci/mmol), and 2 mM myosin light chain kinase at 25 °C. Unless otherwise indicated, 10 μM CaM was included in the reaction. The assay was initiated by addition of the kinase.

**RESULTS**

**Construction and Expression of the Bacterial Plasmid Containing MLCK cDNA**—In order to achieve high level expression of MLCK cDNA in bacteria, the plasmid vector pOTS (18) was used. This vector is under the control of a λ phage promoter inducible by nalidixic acid. To construct the expression plasmid, a 1456-bp fragment from MLCK cDNA MK2.1 was inserted into the BamHI site of the vector. This fragment encoded the putative catalytic and calmodulin-binding domains of the smooth muscle enzyme. Shown in Fig. 1 are the resultant construct, designated pOTS-MK1.4, containing a portion of the smooth muscle MLCK cDNA (Asp'47–Glu'108)
Bacterial Expression of MLCK

found in the protein obtained in the 40–60% ammonium sulfate fraction. After dialysis this protein fraction was applied to a DEAE-cellulose column pre-equilibrated with a buffer containing 20 mM NaCl. The column was extensively washed with the same buffer and then eluted with a NaCl gradient. The flow-through and the eluted fractions were assayed for MLCK activity. The majority of MLCK activity eluted between 150- and 300 mM NaCl. No detectable activity was observed in the flow-through fraction. The active fractions were pooled, dialyzed to remove salt, and loaded onto a heparin-Sepharose column equilibrated with a buffer containing 50 mM NaCl. The flow-through fraction did not contain any MLCK activity. The bound proteins were eluted from the column with a 50–500 mM NaCl gradient. The peak of the MLCK activity eluted at 250–300 mM NaCl. The active fractions were pooled and dialyzed against a buffer containing 50 mM NaCl and 1 mM CaCl₂ prior to loading on a CaM-Sepharose 4B affinity column. After extensive washing with the same buffer, bound protein was eluted by the addition of 50 mM NaCl and 1 mM EGTA. More than 90% of the protein applied to this column was recovered in the flow-through fraction. Kinase activity was monitored in both flowthrough and eluted fractions. Only the fractions eluted with EGTA showed MLCK activity. Table I summarizes the purification of the expressed MLCK protein at the various steps of purification. Between 0.5 and 1.0 mg of bacterially synthesized kinase could be isolated from 1 liter of the culture.

**Characterization of the MLCK Protein Expressed in E. coli**—The bacterially expressed protein purified as described was characterized by (i) gel electrophoresis, (ii) CaM binding, (iii) immunoreactivity to antibodies against chicken gizzard MLC, and (iv) ability to phosphorylate both native myosin and isolated myosin light chains. The polypeptide composition of the purified protein fraction was examined by SDS-PAGE. The expression of the 1456-bp cDNA insert should produce a 50-kDa protein. Fig. 2 (lane 1) shows the most prominent protein, representing about 80% of the total assessed by densitometry, is approximately 50 kDa. The SDS-PAGE fractionated proteins were transferred to nitrocellulose and immunoblotted with affinity purified antibody to chicken gizzard MLCK. As shown in Fig. 2 (lane 2), the antibody reacted only with the 50-kDa polypeptide. In a control experiment, an aliquot from pOTS-transformed bacterial cell extract was subjected to similar immunoblot analysis. None of the polypeptides in the control extract reacted with the anti-MLCK antibody (data not shown). These data reveal that the pOTS-MK1.4 construct produces an appropriately sized immunoreactive protein.

The purified protein fraction was tested for CaM binding using a gel overlay assay. As shown in Fig. 2 (lane 4), the same 50-kDa polypeptide which reacted with anti-MLCK antibody exhibited binding to ³²P-labeled CaM. This binding of CaM was entirely Ca²⁺-dependent (Fig. 2, lane 3). Extracts prepared form pOTS-transformed bacteria did not show any CaM binding in similar overlay assays (data not shown).

<table>
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<td>Purification of bacterially expressed MLCK (MK-50K)</td>
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<tr>
<td>Fraction</td>
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</tr>
<tr>
<td>40–60% ammonium sulfate fraction</td>
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<tr>
<td>Heparin-Sepharose</td>
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<td>Heparin-Sepharose</td>
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<td>CaM affinity column</td>
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* From 1 liter of bacterial culture.
was found to phosphorylate only the 20-kDa MLC whether peptide isolated from bacteria.

possesses the characteristics of the authentic chicken gizzard calcium, respectively.

When the reactions were separated on SDS-PAGE and analyzed by autoradiography, the bacterially produced enzyme was phosphorylated by (as described in the text) in the presence of EGTA and calcium, respectively. Lanes and represent the autoradiograms of myosin light chains phosphorylated by MK-50K and $\gamma[^{32}P]$ATP as described under "Experimental Procedures" using native myosin or isolated light chains, respectively, as substrates.

In order to investigate whether the bacterially expressed 50-kDa enzyme could phosphorylate specifically the 20-kDa myosin light chains, kinase assays were performed using either native gizzard myosin or isolated MLC as substrate. When the reactions were separated on SDS-PAGE and analyzed by autoradiography, the bacterially produced enzyme was found to phosphorylate only the 20-kDa MLC whether native myosin (Fig. 2, lane 5) or isolated MLC (Fig. 2, lane 6) were used as substrates. These data establish that the bacterially expressed 50-kDa MLCK peptide (termed MK-50K) possesses the characteristics of the authentic chicken gizzard MLCK protein.

**Bacterial Expression of MK1.4 Mutants and Characterization of the Resulting Proteins**—The pseudosubstrate and CaM-binding region of MLCK extends from Ala-484 to Ser-512 based on the amino acids encoded by MK 2.1 (3). The MK-50K protein contains approximately 100 amino acids COOH-terminal of Ser-512. The MLCK cDNA contains a unique NcoI recognition site (CCATGG) at Met-513 (see Fig. 1). To determine if the removal of these additional 100 amino acids affected the properties of bacterially produced MLCK, the pOTSM-K1.4 plasmid was linearized with NcoI, digested with S1 nuclease to create blunt ends, and recircularized by ligation. These manipulations result in removal of 360 nucleotides from the COOH terminus of MK1.4 and created an in-frame stop codon after the codon for Ser-512, Nalidixic acid induction of this new construct, pOTSM-K1.1, resulted in production of a 40-kDa protein. This enzyme fragment was purified as described for MK-50K and analyzed by SDS-PAGE (Fig. 2, lane 7). As shown in Fig. 2 (lane 8), only a 40-kDa polypeptide (termed MK-40K) reacted with MLCK antibodies. The molecular size of this protein is consistent with the predicted size of the protein product of MK1.4. This polypeptide represented approximately 70% of the protein in the purified fraction.

The CaM-binding and CaM-dependent kinase activities of MK-50K and MK-40K were compared with those of the authentic chicken gizzard MLCK. Equivalent moles of these molecular forms of MLCK based on the densitometric scans of Coomassie Blue-stained SDS gels, were used in both assays. Fig. 3A shows CaM binding as a function of enzyme protein concentration. Calmodulin binding increases in a linear fashion in response to elevated MLCK protein. The curves are very similar regardless of the enzyme form utilized as a CaM-binding protein. Fig. 3B depicts the enzyme activity of the three forms of MLCK as a function of CaM concentration using isolated MLC as a substrate. The Kₘ values for MK-50K and MK-40K were approximately 5 nM compared to approximately 2 nM for the 130-kDa gizzard native enzyme as determined in our laboratory under the conditions listed under "Experimental Procedures." The turn-over numbers for the 130- and 40-kDa proteins were 700 mol of phosphate/min/mol and 480 mol of phosphate/min/mol of enzyme, respectively. These results show that the CaM-dependent kinase activity of the authentic MLCK is retained in the truncated 40-kDa peptide.

A series of deletion mutations were then prepared in pOTSM-K1.4 in order to evaluate the nature of amino acids involved in the CaM binding and catalytic domains. pOTSM-K1.4 was linearized with NcoI at the codon for Ser-512, which constitutes the last amino acid of the MK-40K fragment. A series of 3'-deletion mutants was obtained by digestion with Exo-
nuclease III. The exonuclease reaction was slowed by using a high salt concentration and low temperature in order to better control the progression of deletions. The mutants were sequenced to determine the extent and 3' end of each deletion. In many mutants, exonuclease digestion followed by out-of-frame ligation created artificial translational stop codons immediately following the point of deletion. Only these mutants were selected to be tested for ability to produce truncated forms of MLCK in bacteria. In addition, a Kinase(-) mutant was constructed by utilizing two convenient SSP1 restriction sites in the pOTS-MK1.4 plasmid. This mutant harbored a gross deletion of 300 nucleotides in the catalytic region while the CaM-binding region remained intact. The various mutant proteins were partially purified from bacterial extracts fractionation on a DEAE-cellulose column as described before. The bound proteins in each case were eluted from the DEAE-cellulose column in a stepwise manner with 0.4 M salt. Expression of each mutant protein was confirmed by immunoblot analysis of the DEAE-purified fraction with anti-MLCK antibody (Fig. 4A). In all cases the molecular size of the mutant proteins was consistent with the coding size of their respective DNAs. The CaM binding activity of each mutant was analyzed by gel overlay assay as shown in Fig. 4B. In addition, all of the mutant proteins were tested for MLCK activity using MLC as a substrate (Fig. 5). Kinase activity was also evaluated after limited proteolysis by trypsin. Protease activity was inhibited by the addition of soybean trypsin inhibitor prior to the kinase assay. As summarized in Fig. 5, only two proteins, MK-40K (the product of pOTS-MK1.1) and the protein product of the Kinase(-) construct, bound CaM under the conditions used. As expected, the Kinase(-) mutant protein did not exhibit MLCK activity although it still bound CaM. Deletion of amino acids from Ser-512 to Ile-507, Arg-509, Gly-501, or Thr-500 resulted in constitutively active enzymes. Further deletions to Lys-496, Lys-482, Lys-476, or Cys-462 also failed to produce active enzyme. However, Ca2+/CaM-independent activity was obtained following trypsin treatment. The Ca2+/CaM-independent kinase activity of all proteolytically activated mutant proteins could be inhibited by the pseudosubstrate prototope peptide Ala480-Gly500 (data not shown). However, mutants that terminated within the catalytic homology region (at Lys-445 and Trp-436, respectively) yielded inactive enzymes that could not be activated by limited proteolysis. To evaluate the possibility that inhibitory proteins in the bacterial extract repress MLCK activity and are removed upon trypsin treatment, we carried out further purification of two of the mutant proteins. The products terminating at Lys-476 and Lys-445 were passed through DEAE-cellulose and heparin-Sepharose columns as described under “Experimental Procedures.” In both cases, the partially purified proteins exhibited similar characteristics to the protein present in crude extracts (data not shown). These results demonstrate that expression of truncated forms of the MLCK devoid of the pseudosubstrate region do not necessarily result in constitutively active enzyme.

**DISCUSSION**

The estimated $M_r$ of the chicken smooth muscle form of MLCK is 130,000. We have successfully produced a 40-kDa fragment of this enzyme in a bacterial expression vector. The purified fragment exhibits similar but not identical CaM binding and kinase properties to the native enzyme. The 40-kDa fragment termed MK-40K, is truncated at its COOH-end at Ser-512 which represents the terminal amino acid of this enzyme, this residue is followed COOH-terminal by a further 157 amino acids. The 40-kDa mutant is therefore similar to the rabbit skeletal muscle enzyme that terminates very...
close to the end of the CaM binding domain (33, 34). Our results demonstrate that the COOH-terminal extension of the gizzard protein is not essential for either enzymatic or calmodulin regulatory properties. Interestingly, this region of the cDNA hybridizes to a 2.7-kb mRNA in addition to the 5.5-kb MLCK mRNA (3). We have recently noted that the 2.7-kb mRNA is unique and can encode a protein that initiates at Met-513, consists of the identical 156 amino acids that occur in MLCK and utilizes the same TGA termination codon.\(^2\) The abundance of the 2.7-kb mRNA is regulated by steroid hormones in the chicken oviduct whereas the mRNA for MLCK (5.5 kb) is not (35). Such observations raise the possibility that the COOH-terminal region of gizzard MLCK may endow a separate property or function to the enzyme distinct from the kinase activity. This property or function would not exist in the skeletal muscle form of the enzyme.

The NH\(_2\)-terminal region of smooth muscle MLCK is not essential for catalytic function. Proteolytically derived forms of both the avian smooth muscle and rabbit skeletal muscle enzyme have been reported. These forms have resulted from proteolysis from both ends of the protein (12, 15, 36). MK-40K begins at Asp-147 and terminates at Ser-512. The first Gly of the ATP-binding site is at position 223 (3). Thus the limit size of the protein that would contain all of the necessary information to encode a CaM-dependent MLCK is 31 kDa. Sellers and Harvey (37) isolated the MLCK from the invertebrate Limulus. This enzyme was CaM-dependent and, at 37 kDa, represents the smallest naturally occurring CaM-dependent MLCK reported to date. The Dicystostelium enzyme, which is only slightly smaller than the Limulus form (30 kDa), does not require CaM for activity and is actually inhibited by Ca\(^{2+}\) (38).

Results obtained with COOH-deletion mutants of MK-40K reveal that the removal of only three amino acids from the COOH-end of the CaM binding domain, Gly\(^{306}\)-Arg\(^{309}\)-Leu\(^{310}\), results in an inactive protein that neither binds nor is regulated by CaM. Such a result is consistent with our previous studies (6) which showed that a 64-kDa proteolytic fragment of MLCK that was inactive and did not bind CaM was truncated at Arg-505. When CaM-binding proteins of various number of CaM-dependent enzymes are aligned to show the highest identity, an Arg is frequently found at the equivalent position to Arg-509 in gizzard MLCK (1, 7). Our studies show that the mutation of Arg-509 to Ile abolishes CaM binding. Since the most usual characteristic of a CaM-binding region is a propensity to form an amphipathic helix (39) it is likely that inhibition of CaM binding is due to neutralization of this positive charge. Helical wheel analysis reveals that the positive charge from Arg-509 is centered among the positive charges on one face of the putative \(\alpha\)-helix. Since the equivalent Arg in many CaM-binding peptides occupies a similar position on a helical wheel, it is reasonable to presume that the nature and location of this positive charge is important. Although we attempted to obtain additional substitutions in our mutagenesis procedure, we have failed to do so.

Mutation of Gly-508 to Glu also results in complete inhibition of CaM binding to MLCK. We cannot be certain whether Gly is an essential component of the CaM-binding site or whether it plays a role in a negative charge or a conformational change that prevents the binding. Glycine is not a strong \(\alpha\)-helix forming amino acid and is frequently considered to impart flexibility to an amphipathic structure. If the flexible tether model of CaM bind-

\(^1\) D. Needleman and A. R. Means, unpublished data.

References


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