Definition of the Inhibitory Domain of Smooth Muscle Myosin Light Chain Kinase by Site-Directed Mutagenesis†

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ABSTRACT: Site-directed mutagenesis of smooth muscle myosin light chain kinase was applied to define its autoinhibitory domain. Mutants were all initiated at Leu-447 but contained varying lengths of C-terminal sequence. Those containing the complete C-terminal sequence to Glu-972 possessed kinase activities that were calmodulin-dependent. Removal of the putative inhibitory domain by truncation to Thr-778 resulted in generation of a constitutively active (calmodulin-independent) species. Thus, the inhibitory domain lies to the C-terminal side of Thr-778. Truncation to Lys-793 and to Trp-800 also resulted in constitutively active mutants, although the specific activity of the latter was less than the other mutants. None of the truncated mutants bound calmodulin. For each mutant, the K_m values with respect to ATP and to the 20 000-dalton light chain were similar to values obtained with the native enzyme. The presence of the inhibitory domain was detected by activation of kinase activity following limited proteolysis with trypsin. Using this procedure, it was determined that the inhibitory domain was manifest only in the mutant truncated to Trp-800 and was absent from that ending at Lys-793. These results indicate that a critical region of the inhibitory domain is contained within the sequence Tyr-794 to Trp-800. This region overlaps with the calmodulin-binding site for five residues. Our assignment of the inhibitory sequence is consistent with autoinhibition via a pseudosubstrate domain.

Myosin light chain kinase (MLCK)[1] is a key enzyme in the regulation of contractile activity in smooth muscle. Phosphorylation of Ser-19 on each of the two 20 000-dalton light chains by MLCK is thought to be essential for the initiation of contraction (Hartshorne, 1987). This event is coupled to the transients in intracellular Ca^{2+} via CaM, which as the Ca^{2+}–CaM complex is essential for activation of MLCK. Dephosphorylation of myosin is involved in the relaxation process.

Because of its central role in the contractile cycle of smooth muscle, and possibly in motile processes of nonmuscle cells (Stull et al., 1986), there have been several studies characterizing the MLCK molecule. Various models were proposed in which distinct regions, or domains, are defined (Hartshorne, 1987). Identification of functional domains was facilitated by sequences derived for gizzard MLCK from a cloned partial cDNA (Guerriero et al., 1986) and, more recently, the complete cDNA (Olson et al., 1990). Complete sequences for the rabbit (Takio et al., 1986) and rat (Roush et al., 1988) skeletal muscle enzymes also have been reported. From these studies, a general pattern emerged of a central active site flanked on its C-terminal side by the CaM-binding site.

An interesting question for CaM-dependent enzymes in general is how activity is regulated via interaction with Ca^{2+}–CaM. For skeletal muscle MLCK, the inactivity of the apoenzyme was suggested to reflect internal inhibition by an inhibitory domain (Edelmann et al., 1985). For the gizzard MLCK, it was proposed that inhibition is due to the interaction of the active site with a pseudosubstrate region and that this interaction is dissociated by the binding of Ca^{2+}–CaM (Kemp et al., 1987). This hypothesis is based on the juxtaposition of basic residues of the light-chain phosphorylation site with a stretch of the MLCK sequence, aligning His-805 of MLCK [residue assignment taken from the complete cDNA sequence (Olson et al., 1990)] with Ser-19 of the light chain. Extension of the light-chain sequence to the N-terminus gives a homologous MLCK peptide beginning at Ser-787. With this alignment, several basic residues match; for MLCK, residues 802, 799, 798, 797, 792, and 790 line up with light-chain residues 16, 13, 12, 11, 6, and 4, respectively. The pseudosubstrate concept was supported by the finding that the synthetic peptide 783–804 inhibited MLCK activity even at saturating CaM concentrations (Kemp et al., 1987). In addition, it was shown (Ikebe et al., 1987) that tryptic hydrolysis of the gizzard apoenzyme yielded initially an inactive fragment of M_r ~64 000, followed by a CaM-independent, active fragment of M_r ~61 000. The generation of activity by the removal of the 3000 molecular weight peptide is consistent with the loss of the pseudosubstrate domain.

The boundaries of the pseudosubstrate domain are not established. Inhibition of the constitutively active MLCK fragment by synthetic peptides showed that the peptide Ala-783 to Gly-804 was a more potent inhibitor than the peptide Ala-783 to Ala-796 (Ikebe et al., 1987), and this suggested that the pseudosubstrate region may overlap with the putative CaM-binding site, Ala-796 to Ser-815 (Lukas et al., 1986). Support for this idea came from the determination (Pearson et al., 1988) of Arg-808 as the C-terminal residue of the inactive tryptic fragment. The C-terminus of the active tryptic fragment was shown to be Lys-779, eight residues N-terminal from the pseudosubstrate region, Ser-787 to Val-807 (Olson

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[1] Abbreviations: MLCK, myosin light chain kinase; CaM, calmodulin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Bio-CaM, (biotinyl-N^{α}-aminocaproyl)calmodulin; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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et al., 1990). Studies with other proteases (Ito et al., 1989a) have confirmed that the conversion from the inactive to active fragment is due to cleavage only at the C-terminal end of the molecule. In addition, the C-terminal residue of the inactive fragment generated by endoproteinase Lys-C was determined as Lys-802. Thus, by application of limited proteolysis, the two margins of the pseudosubstrate, or inhibitory sequence, can be defined as Lys-779 and Lys-802.

Recently, it was suggested that the inhibitory sequence is Asp-777 to Lys-793 (Ikebe et al., 1989). Although this is close to the putative pseudosubstrate sequence, and in fact overlaps for seven residues, it is sufficiently different to challenge the pseudosubstrate concept. In order to resolve this controversy and to identify some of the residues important in the regulation of activity, we have constructed several mutants of MLCK. Obviously, the area of MLCK that was targeted was the C-terminal part of the molecule, and this incorporated those regions suggested as important for the regulation of activity.

Materials and Methods

Protein Preparations and Procedures. Myosin light chain kinase was prepared from frozen turkey gizzard by the procedure of Ikebe et al. (1987). Kinase activity of expressed proteins was monitored (Walsh et al., 1983) by using either turkey gizzard myosin (Ikebe & Hartshorne, 1985) at 1 mg/mL or mixed myosin light chains (Hathaway & Haeberle, 1983) at 0.8 mg/mL, with 0.2–0.5 mM [γ-^{32}P]ATP, 1 mM $MgCl_2$, 85 mM KCl, 30 mM Tris-HCl (pH 7.5), and either 0.1 mM $CaCl_2$ plus 5 μg/mL CaM or 1 mM EGTA. For determination of the K_m value for substrate, the 20 000-dalton light chain was purified on phenyl-Sepharose (Hathaway & Haeberle, 1983), and traces of CaM were removed by a melittin affinity column [melittin coupled to Affi-Gel 10 (Bio-Rad)]. CaM was isolated from frozen beef testes (Walsh et al., 1983). Preparation of (biotinyl-N^{ϵ}-aminocaproyl)calmodulin (Bio-CaM) and application to nitrocellulose blots were as described by Kincaid et al. (1988). The CaM affinity column was prepared by using Affi-Gel 15 (Bio-Rad). SDS–PAGE was carried out on 7.5–20% polyacrylamide gradient slab gels using the discontinuous buffer system of Laemmli (1970). Molecular weights were estimated from standards in the MW-SDS-200 kit (Sigma). Polyclonal antibodies to turkey gizzard MLCK were raised in rabbits (Guerriero et al., 1981), and conditions for Western blots were as given earlier (Ito et al., 1989b).

MLCK Expression Vector Construction. Myosin light chain kinase mutants were expressed in bacteria using the expression vectors pRIT2T (Pharmacia LKB Biotechnology) or pET-3a (Studier et al., 1990). The vector pRIT2T contains a fragment coding for protein A, which has IgG-binding activity, followed by the pUC9 polylinker region for the insertion of cDNAs. A cDNA inserted in the proper reading frame and orientation will be expressed as a fusion protein that contains the IgG portion of protein A. The first construct was made by digesting pMK 2.1 (Guerriero et al., 1986) with *PstI* to remove the region coding for amino acids 447–972. This fragment includes the regions predicted to contain calmodulin binding, pseudosubstrate, catalytic activity, and phosphorylation sites. The fragment was isolated by electrophoresis and extraction from low-melting agarose. The vector was digested with *PstI*, and the 5′ termini were dephosphorylated by using calf intestinal phosphatase. The insert and vector were ligated by using T4 ligase, and transformation was performed by the method of Hanahan (1983) in *Escherichia coli* strain N99cI. Transformants were selected and analyzed by restriction analysis for proper orientation. This construct is referred to as pMK.Glu-972 (the last amino acid and number of that residue are used in identifying these plasmids). The next construct was made by digesting pMK 2.1 with *Bst*XI and *Nco*I which removes the region coding for amino acids Asn-780 to Ser-815, i.e., that sequence containing the regulatory domain. The 3′ overhang and one additional base were removed by S1 nuclease digestion. The *Nco*I end was filled by using the Klenow fragment of DNA polymerase I and ligated to the blunt-end *Bst*XI site. The resulting construct is an open reading frame to Thr-778 followed by three additional amino acids, Thr-Trp-Gln, and then a termination codon. This construct is referred to as pMK.Thr-778. The construct pET.Glu-972 was created by digesting the plasmid pMK.Glu-972 with *Bam*HI and removing the MLCK insert which was subcloned into the *Bam*HI site of the expression vector pET3-a. Expression in this vector is regulated by T7 polymerase and induced by isopropyl β-D-thiogalactoside.

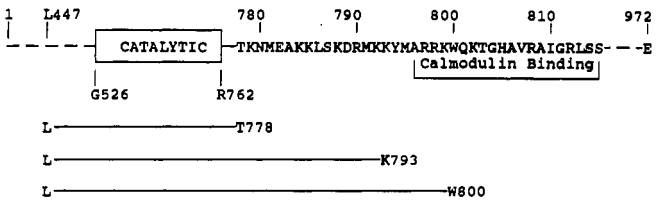

FIGURE 1: Diagrammatic representation of the MLCK molecule indicating the truncation points of the mutants used. Each mutant was initiated at Leu-447. For the pMK mutants, a fragment of protein A was fused N-terminal to Leu-447. The pseudosubstrate domain extends from Ser-787 to His-805.

Site-Directed Mutagenesis. A diagrammatic representation for the design of the mutants is given in Figure 1. The sites of truncation are shown relative to the active site, the pseudosubstrate domain, and the CaM-binding site. Two constructs were derived from pMK.Glu-972 by using site-directed mutagenesis to create termination codons. The region coding for amino acids 492–972 was subcloned into the phagmid pBS+ (Strategene Cloning Systems) and was used to make single-stranded DNA (Kunkel, 1985) for site-directed mutagenesis (Zoller & Smith, 1983). Complimentary oligonucleotides were made (University of Arizona Macromolecular Structures Facility) with single mismatches to serve as primers: 5′-GAAGAAATAGATGGCCAGA-3′ for pMK.Lys-793, 5′AGAAAATGGTAGAAAACAG-3′ for pMK.Trp-800, 5′-AAACTTTCCTAAGAT-AGGA-3′ for pMK.Ser-787, 5′-CATGCTGTCTGAGCAATAGGA-3′ for pMK. Val-807, and 5′-CTGTCATCCTAGGCAATGAT-3′ for pMK.Ser-815. All mutants were sequenced (Sanger et al., 1977) to confirm changes. The mutated regions were subcloned into pMK.Glu-972 for expression of the proteins.

Expression of MLCK in Bacteria. Bacteria (*E. coli* strain N4830-1) with inserts in pRIT2T were cultured in LM medium (3-61) overnight at 30 °C with continuous shaking. An equal volume of LM medium at 54 °C was added and incubation continued for 90 min at 42 °C. For expression in pET-3a, BL21(DE3) cells containing pLysS were grown in LM containing 50 μg/mL ampicillin and 30 μg/mL chloramphenicol. When the cell culture reached an OD_{600} of 0.6–1.0, the culture was made 0.4 mM in isopropyl thiogalactoside and harvested after 3 h of induction.

Cells were collected by centrifugation at 7000*g* for 20 min, and the pellet was washed with 20 volumes of 40 mM Tris-HCl (pH 8.0). After centrifugation for 20 min, the pellet was extracted with 100–200 mL of 30 mM Tris-HCl (pH 7.5), 30 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM diisopropyl fluorophosphate, 10 μg/mL trypsin inhibitor, 10 μg/mL leupeptin, 5% glycerol, and 0.5 mg/mL lysozyme (for

Table I: Specific Activities and Enzymatic Parameters of MLCK Mutants

MLCK mutant	specific activity[a] +Ca^{2+}	specific activity[a] −Ca^{2+}	K_m(CaM) (nM)	K_m(LC20) (μM)	K_m(ATP) (μM)
pMK.Glu-972	660 ± 17	0.8 ± 0.1	23	25	71
pET.Glu-972	790 ± 23	0.9 ± 0.2	33	31	67
pMK.Trp-800	96 ± 5	104 ± 2		17	91
pMK.Lys-793	355 ± 15	400 ± 22		29	83
pMK.Thr-778	244 ± 6	239 ± 19		20	80
native MLCK[b,c]	(10–30) × 10^3		1–2	5–14	50–68

[a] Specific activities (nanomoles of P_i transferred per minute per milligram) were calculated on the basis of total protein in the preparation. Values given are the average ±SD from at least for determinations. [b] Data for gizzard MLCK taken from Walsh and Hartshorne (1982) and Stull et al. (1986). [c] Published values for V_{max} vary considerably. In general, activities are higher if isolated light chains, compared to intact myosin, are used.

pMK.Glu-972 and pET.Glu-972) or with this solution with 1 mM EGTA and 5 mM EDTA minus the dithiothreitol (for the other mutants). The mixture was stirred for 15 min at 4 °C and sonicated with 3 × 30 s bursts (Branson sonifier, setting 30–40), homogenized with a glass–glass Potter-type homogenizer, and centrifuged at 100000*g* for 1 h. For pMK.Glu 972 and pET.Glu 972, $CaCl_2$ was added to the supernatant to 1.2 mM and applied to a CaM affinity column [1 × 6 cm (Walsh et al., 1982)] equilibrated with 30 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM $CaCl_2$, 1 mM dithiothreitol, and 5% glycerol. Bound protein was eluted with this solvent containing 2 mM EGTA. This fraction was used as the partially purified pMK.Glu 972. For pMK mutants, the supernatant was applied to a IgG_1 affinity column [Affi-Gel 10, 14 mL plus 65 mg IgG_1, coupled according to the manufacturer's specifications (Bio-Rad)] equilibrated with 30 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 1 mM EDTA, 1 mM EGTA, and 5% glycerol. Bound protein was eluted with 0.1 M sodium citrate hydrochloride (pH 6.0), 1 mM EDTA, and 5% glycerol, neutralized (to pH 7.5) with Tris base and dithiothreitol added to 1 mM. This fraction was used as partially purified MLCK mutants.

Results

The five mutants of MLCK that were focused on were pMK.Glu-972, pET.Glu-972, pMK.Thr-778, pMK.Trp-800, and pMK.Lys-793. Representative SDS–PAGE profiles for partially purified preparations are shown in Figure 2. Molecular weights calculated from the sequence (Olson et al., 1990) for these mutants are 87.3K, 58.3K, 65.7K, 68.2K, and 67.4K, respectively. Molecular weights estimated from SDS–PAGE were 102K, 77K, 64K, 67K, and 66K, respectively. It is known that the molecular weight of MLCK as estimated by SDS–PAGE is anomalously high, 130K compared to 107.5K (Olson et al., 1990). This is due predominantly to the acidic C-terminal part of MLCK, i.e., telokin (Ito et al., 1989c), and mutants without this segment have similar values for the calculated (from sequence) and estimated (from SDS–PAGE) molecular weights. The percentage of these components in the partially purified preparations varied between 20 and 30% of the total protein. The identity of these components as MLCK mutants was established by Western blots, using a polyclonal antibody to MLCK (Figure 2), and, where possible (only for pMK.Glu-972 and pET.Glu-972), the CaM-binding domain was detected by using Bio-CaM (Figure 2). Binding of CaM was not detected for pMK.Trp-800 (Figure 2). Slight proteolysis of these preparations was evident from the Western blots and Bio-Cam staining patterns. In spite of the use of several protease inhibitors, this was unavoidable.

The specific activities (toward isolated myosin light chain) and some enzymatic parameters are shown in Table I. The two mutants with the largest MLCK inserts, pMK.Glu-972

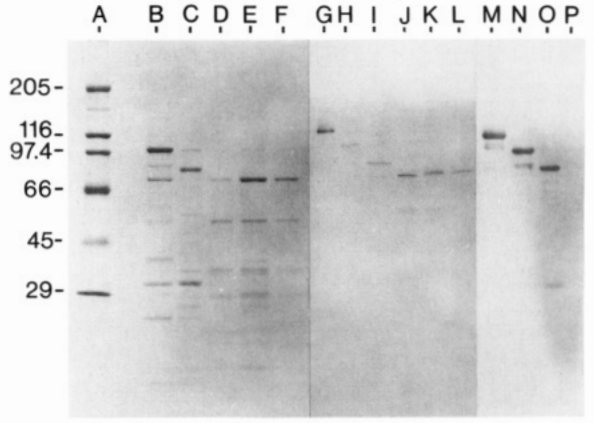

FIGURE 2: Characterization of the MLCK mutant preparations. Lanes A–F show SDS–PAGE patterns. Lanes G–L, Western blots using a polyclonal antibody to turkey gizzard MLCK. Lanes M–P, staining pattern with Bio-CaM. Lane A, molecular weight markers. Lanes B, H, and N, pMK.Glu-972. Lanes C, I, and O, pET.Glu-972. Lanes D and J, pMK.Thr-778. Lanes E and K, pMK.Lys-793. Lanes F, L, and P, pMK.Trp-800. Lanes G and M, native MLCK.

and pET.Glu-972, contained the active site plus all of the sequence to its C-terminal side. These were predicted to be CaM-dependent, and clearly they met these expectations. Both mutants showed a Ca^{2+}–CaM dependency similar to the native enzyme. The specific activities are reasonable if the purity of approximately 30% is taken into account. The point of expressing both mutants was to determine if fusion with the protein A insert affected enzymatic properties. With the limited data available, it appears that the protein A segment has no detectable effect. For both mutants, the K_m values for the light-chain substrate and for ATP are similar to the native MLCK (see Table I).

The affinity of CaM for the pertinent mutants was reduced compared to the native enzyme (Table I). There is no explanation for the higher K_m values obtained with the mutants. Most of the enzymatic assays, however, were carried with excess CaM (at approximately 280 μM), and the reduced affinity of the mutants for CaM should not influence the levels of observed kinase activity.

The other three mutants are truncated on the C-terminal side of the active site, and each has lost the sequence thought to be required for interaction with CaM, i.e., 796–815. None of these mutants were retained by a CaM affinity column nor did they bind to Bio-CaM. The specific activities of these mutants were not altered by the Ca^{2+}–CaM complex. These data confirm that the interaction with CaM requires residues C-terminal to Trp-800.

The specific activities of the three truncated mutants (pMK.Trp-800, pMK.Lys-793, and pMK.Thr-778) were variable, and were less than the activities of the two longer mutants. These variations did not reflect different concentrations of kinase in each preparation since the proportion of

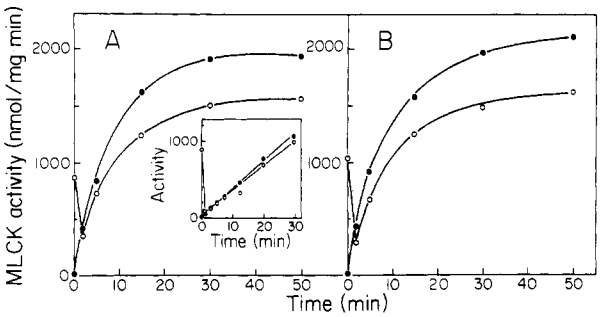

FIGURE 3: Proteolysis by trypsin of pMK.Glu-972 (A) and pET.Glu-972 (B). Mutants (approximately 0.2 mg/mL) were digested at 0 °C with trypsin (80 μg/mL), aliquots were withdrawn at indicated times, soybean trypsin inhibitor was added (10 × weight excess to trypsin), and activity was determined (see Materials and Methods) with Ca^{2+} and CaM (○) and with EGTA (●) using isolated light chains. For the inset in (A), trypsin was reduced to 15 μg/mL; other conditions were the same.

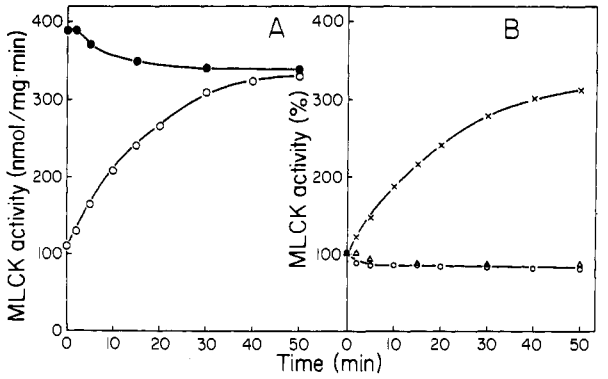

FIGURE 4: Proteolysis by trypsin of truncated MLCK mutants. (A) Proteolysis of pMK.Lys-793 (●) and pMK.Trp-800 (○). (B) Effect of proteolysis, expressed as a percentage of original activity, on pMK.Trp-800 (×), pMK.Ser-787 (△), and pMK.Lys-793 (○). Assays were carried out in EGTA using isolated light chains (see legend to Figure 3 and Materials and Methods).

the kinase component was almost identical (as judged from Western blots and scans of the SDS–PAGE profiles). The specific activity of pMK.Trp-800 was considerably lower than those of pMK.Thr-778 and pMK.Lys-793 but was nevertheless too high to allow an unequivocal decision about the presence or absence of the inhibitory sequence. In spite of extensive truncation at both the C-terminal and N-terminal parts of the molecule (each mutant begins at Leu-447), the interactions of these mutants with both light chain and ATP are not affected significantly. The K_m values are essentially the same for each mutant and do not differ markedly from those of the native enzyme.

With the three truncated mutants, the parts of the MLCK molecule that were deleted were chosen as a test of the pseudosubstrate hypothesis. These mutants either lacked the pseudosubstrate sequence, pMK.Thr-778, or contained short, pMK.Lys-793, or longer, pMK.Trp-800, segments of this sequence. If the pseudosubstrate hypothesis is accepted, it would be predicted that the former two mutants are constitutively active and the latter is, at least partially, inactive. Since each of the expressed MLCK mutants contained measurable kinase activity, another criterion was necessary to establish the presence or absence of an inhibitory sequence. It was shown previously that trypsin converts an inactive to a constitutively active fragment by removal of an inhibitory region (Ikebe et al., 1987). Limited proteolysis with trypsin was therefore used to evaluate each of the mutants for the presence of an inhibitory sequence. This procedure is illustrated using the two mutants known to possess the complete inhibitory domain, i.e., pMK.- and pET.Glu-972. For each mutant, proteolysis by trypsin produced similar results as shown in Figure 3. The initial phase of proteolysis caused a loss of Ca^{2+}–CaM-dependent activity, as shown best by the inset of Figure 3A, followed by the generation of CaM-independent activity. These mutants behaved identically with the native kinase. The different phases of this activity profile thus were assumed to represent, initially, the removal of the CaM-binding site and, subsequently, removal of the inhibitory sequence. For each of the mutants that exhibited activation by trypsin, the constitutively active kinase activity was higher in the presence of EGTA, compared to the presence of Ca^{2+}. The $[Ca^{2+}]$ dependence of this effect was relatively high, and the Ca^{2+} concentration required for half-maximal inhibition was 0.3 mM.

The two critical mutants to analyze by this procedure were pMK.Lys-793 and pMK.Trp-800. As shown in Figure 4A, proteolysis of these mutants caused different effects. In the case of pMK.Trp-800, an activation was observed, and for pMK.Lys-793, the kinase activity was inhibited slightly. (That pMK.Lys-793 indeed was cleaved by trypsin was confirmed by SDS–PAGE.) Proteolysis of pMK.Thr-778 gave the same results as pMK.Lys-793 (data not shown). These effects are more dramatically illustrated in Figure 4B as percentages of original activities. Also shown in this figure are results from another mutant, pMK.Ser-787. On the basis of these results, it is proposed that the mutant pMK.Trp-800 contains an inhibitory sequence that is removed by proteolysis and that pMK.Ser-787 and pMK.Lys-793 are not subject to autoinhibition and do not contain the inhibitory sequence. With respect to the latter, it may therefore be concluded that a critical region of the molecule is contained within the sequence Tyr-794 to Trp-800.

DISCUSSION

The objective of this study was to obtain additional information on the structure–function relationships of smooth muscle MLCK and in particular to examine the phenomenon of autoinhibition in the apoenzyme. Previous studies (outlined in the introduction) defined the inhibitory sequence within the boundaries Lys-779 and Lys-802. Since this sequence effectively contained both the pseudosubstrate domain (Kemp et al., 1987) and the inhibitory sequence proposed by Ikebe et al. (1989), additional data were required to evaluate the two hypotheses. This was achieved by constructing and expressing mutants of MLCK in *E. coli*.

Other studies had found that various mutants of smooth muscle (Bagchi et al., 1989) and skeletal muscle MLCK (Herring et al., 1990b) were expressed in an inactive form, although activity was observed following tryptic hydrolysis of the smooth muscle mutants (Bagchi et al., 1989). Some of the mutants studied by Bagchi et al. (1989) would be predicted as constitutively active forms of the enzyme, i.e., those truncated to Lys-776 and Lys-779. This was not observed, and it was suggested (Bagchi et al., 1989) that the lack of activity reflected either incorrect folding of the expressed polypeptide or the presence of an additional inhibitory sequence toward the N-terminal part of the molecule involving residues 450–464. One of the mutants expressed in our study contained the sequence Leu-447 to Thr-778. This was constitutively active, in contrast to mutants 450–779 of Bagchi et al. (1989). Thus, it is unlikely that a second inhibitory region exists in MLCK. The most likely explanation for the lack of activity in the truncated mutants obtained earlier is that the expressed protein is not folded correctly. It is not clear why in our hands

the truncated mutants were active, but it was a consistent observation that kinase activity was not detected unless the preparations were exposed to relatively high concentrations of dithiothreitol (see Materials and Methods). One possibility is that disulfide bonds are formed in the expressed kinases that are distinct from those present in the native enzyme. In addition, it was observed that those mutants incorporating the C-terminal part of the molecule to Glu-972 possessed higher kinase activity than the truncated forms. Thus, it is possible that the CaM-binding site and/or telokin are important for the stability of MLCK. Kennelly et al. (1990) found that the calmodulin-binding domain stabilized the apoenzyme of skeletal muscle MLCK, and it was also suggested that a cation-binding site of low affinity might be involved in stabilizing kinase activity. Such has not been established for smooth muscle MLCK, but the persistent observation that the activation of kinase activity induced by proteolysis was higher in EGTA compared to $Mg^{2+} + Ca^{2+}$ may indicate an interaction of Ca^{2+} and MLCK. Other possible explanations for this effect include the presence of a significant proportion of Ca^{2+}–ATP complex or binding of Ca^{2+} to the light chains.

The kinetic parameters of the mutant kinases are similar to those of the native enzyme, with the possible exception of CaM binding which is reduced. The K_m for light chains compared to the native enzyme is within experimental error, and the K_m's for ATP are indistinguishable for the native and mutant enzymes. Thus, it appears that neither the N- nor the C-terminal ends of MLCK have dominant roles in enzymatic properties. This confirms several earlier results with proteolytic fragments of MLCK. With respect to the light-chain binding, it has been proposed recently for the skeletal muscle MLCK that the acidic residues 269 and 270 are important in enzyme–substrate binding (Herring et al., 1990a). Asp-270 is 31 residues to the N-terminal side of the active site (Takio et al., 1986). For the smooth muscle MLCK, there is an acidic cluster, Asp-494, Asp-495, and Glu-496, at approximately the same position with respect to the active site (Olson et al., 1990), and this could be involved in light-chain binding. Accepting this, it is not unreasonable that the K_m values for light chains are similar for the mutants and the native enzyme since this acidic cluster is included in all of the mutants tested in our studies. Herring et al. (1990b) also suggested that residues 165–173 of skeletal muscle MLCK may be important for interaction with the substrate. Since the analogous sequence is missing from our mutants, it is possible that this region is not as dominant for binding affinity as the more C-terminal acidic cluster. Obviously, this is based on extrapolation of our data with the smooth muscle system to results with the skeletal muscle kinase and must be regarded as speculative.

Recently the full-length cDNA of a nonmuscle MLCK has been cloned and sequenced, and several mutants were expressed (Shoemaker et al., 1990). The inhibitory region for the nonmuscle MLCK was defined as residues 1068–1080, and this would correspond to the sequence 783–795 in the gizzard MLCK. Obviously, in the linear sequence, this is close to the region suggested from our studies as important for inhibition of the apoenzyme. However, an important distinction is that in the nonmuscle MLCK the inhibitory and CaM-binding sequences (1082–1101) are proposed as two distinct domains. For the gizzard MLCK, we suggest that the inhibitory and calmodulin-binding sequences overlap for at least five residues. With this arrangement, it is not unreasonable to predict that the binding of CaM would remove the restraints imposed by the inhibitory region.

Each of the three mutants that were truncated at the C-terminal side at residues 778, 793, and 800 were constitutively active and did not bind CaM. Although the specific activity of pMK.Trp-800 was considerably lower than the other two mutants, it was too high to allow an unequivocal decision concerning the presence or absence of the inhibitory region. There are several possible reasons to account for this low level of activity: (a) the inhibitory sequence present in pMK.Trp-800 could be incomplete, and extension to the C-terminal side of residue 800 may provide more complete inhibition. This suggestion is consistent with the recent results of Foster et al. (1990), who identified five regions in the sequence 786–807 that contributed to inhibition. One of these, Lys-802 to Val-807, would not be present in the pMK.Trp-800 mutant; (b) there may be a problem with correct folding of the expressed protein such that complete inhibition was not achieved; and (c) a limited extent of proteolysis could partially activate the preparation. The reason for this low but finite level of activity is not established, but its presence required evaluation of the mutants using an alternative technique, namely, the application of limited proteolysis by trypsin. The validity of this approach has been documented by using native MLCK (Ikebe et al., 1987) and in this study with the regulated MLCK mutants (pET.- and pMK.Glu-972). The only mutant of MLCK to show activation of kinase activity by proteolysis was pMK.Trp-800. On the basis of these results, it is concluded that at least part of the inhibitory region in gizzard MLCK is represented by the sequence Tyr-794-Met-Ala-Arg-Arg-Lys-Trp-800. These seven residues are within the pseudosubstrate sequence in which His-805 is aligned with Ser-19 of the 20000-dalton light chain. Our results, therefore, support the pseudosubstrate hypothesis.

Registry No. MLCK, 51845-53-5; ATP, 56-65-5.

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Temperature Dependence of the Rates of Conformational Changes Reported by Fluorescein 5′-Isothiocyanate Modification of H^+,K^+- and Na^+,K^+-ATPases†

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ABSTRACT: Stopped-flow fluorometry has been used to measure the forward and reverse rates of the conformational change from E_1 to E_2 in the fluorescein-modified proton and sodium pumps (1) as a function of Na^+ and K^+ concentrations to verify the proposed mechanism of ion interaction with the enzymes and (2) as a function of temperature to gain insight into the nature of the conformational transition. (1) The fluorescence changes caused by Na^+ and K^+ are consistent with rapid competitive binding of the two ions to the E_1 conformations of the enzymes followed by rate-limiting transitions between E_1K and E_2K. (2) Reaction coordinate diagrams for the E_1K to E_2K transitions in the H,K-ATPase and Na,K-ATPase are qualitatively similar. Enthalpy barriers to reaction are partially compensated by increased entropy in the transition states. However, there are striking quantitative differences between the two enzymes. The E_2K to E_1K reaction of the H,K-ATPase is more than 2 orders of magnitude faster ($\tau_{1/2}$ = 6 ms at 22 °C) than the reverse rate of the Na,K-ATPase transition ($\tau_{1/2}$ = 1.6 s), explaining repeated failure to detect a K^+-"occluded" form of the H,K-enzyme. The E_2K conformer of the Na,K-ATPase is 3 orders of magnitude more stable than E_1K, while the E_1K and E_2K conformations of the H,K-ATPase are nearly equivalent energetically.

Enzymes that catalyze active transport via formation of a covalent phosphoenzyme intermediate are classified as E_1E_2-type ATPases, where E_1 and E_2 denote different protein conformations that have been postulated to explain coupling of ATP hydrolysis to the physical translocation of ions. Experimental evidence for a conformational change has been obtained by chemical modification of the enzymes with fluorescein 5′-isothiocyanate (FITC),[1] which reacts specifically

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[1] Abbreviations: H,K-ATPase, Mg^{2+}-dependent, H^+-transporting, and K^+-stimulated ATPases (EC 3.6.1.36); Na,K-ATPase, Mg^{2+}-dependent and Na^+- and K^+-stimulated ATPase (EC 3.6.1.37); Ca-ATPase, Ca^{2+}- and Mg^{2+}-dependent ATPase (EC 3.6.1.38); SR, sarcoplasmic reticulum; FITC, fluorescein 5′-isothiocyanate; DCIP, 2,6-dichloroindophenol; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenylcyclohexadienylidene)adenosine 5′-triphosphate; pNPP, *p*-nitrophenyl phosphate; P_i inorganic phosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; SD, standard deviation.

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