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ADP/Vanadate Mediated Photocleavage of Myosin Light Chain Kinase at the Autoinhibitory Region

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The vanadate (Vi)-mediated photocleavage reaction was used to study the interaction between the regulatory segment and the catalytic site of smooth muscle myosin light chain kinase (MLCK). When MLCK was irradiated with long-wave UV (366 nm) in the presence of ADP and Vi, kinase activity was substantially decreased, and the MLCK polypeptide of 130 kDa was cleaved into several smaller fragments with apparent molecular masses of 100, 70, 60, 32, and 28 kDa. Inhibition of kinase activity and photocleavage were both competitively antagonized by the addition of ATP. Inconsistency between the observed maximum levels of UV-induced inhibition of MLCK-mediated phosphorylation (80%) and photocleavage (15-20%) suggested that the photocleavage reaction proceeds as a two-step process. Monoclonal antibodies recognizing the C-terminus of MLCK labeled the 60- and 28-kDa fragments, indicating that MLCK was cleaved at two sites, at 28 and 60 kDa from the C-terminus, within what are believed to be the autoinhibitory region and the catalytic site, respectively. Moreover, Ca^{2+}-calmodulin (Ca^{2+}-CaM) protected against cleavage at the site at 28 kDa from the C-terminus. Analysis of the amino acid composition of the fragment revealed that the cleavage site at 28 kDa from C-terminus occurred at Lys 799±3 amino acid residues, which is in a region where the CaM-binding and pseudosubstrate regions overlap. These results suggest that the three-dimensional structure of MLCK brings the regulatory segment into direct contact with the ATP-binding site. Moreover, the binding of Ca^{2+}-CaM displaces the regulatory segment away from the catalytic site.

Key words: calmodulin, MLCK, photocleavage, regulatory mechanism, vanadate.

Smooth muscle myosin light chain kinase (MLCK) is a Ca^{2+}-calmodulin (CaM)-dependent enzyme that specifically phosphorylates the regulatory light chain of myosin. In smooth muscle and non-muscle cells, MLCK plays an important role in activating actomyosin-based contractility (*1, 2*). The complete amino acid sequence of MLCK has been determined for both skeletal muscle (*3, 4*) and smooth muscle (*5, 6*). In each case, MLCK is composed of a catalytic core homologous to those of other protein kinases and a regulatory segment at the C-terminus, consisting of an autoinhibitory region and a CaM-binding region (*7, 8*).

Autoinhibition in MLCK has been studied by several methods including synthetic peptide inhibition analysis (*9, 10*), limited proteolysis (*11-13*), and truncation of recombinant MLCK (*14, 15*). It has been proposed that the autoinhibitory region, which contains a pseudosubstrate sequence, folds into the active site of MLCK, thereby preventing substrate binding. Ca^{2+}-CaM, in turn, activates MLCK by binding to a site overlapping the autoinhibitory region and causing a conformational change that removes the pseudosubstrate inhibitory region from the active site (*16*). This scenario for CaM-induced enzyme disinhibition has also been proposed for a number of other Ca^{2+}-CaM dependent protein kinases (*10, 17*). Consistent with this notion, information obtained by small-angle X-ray and neutron scattering (*18*), NMR (*19*), and X-ray crystallography (*20*) indicates that the two lobes of CaM come into close contact with and partially envelop the MLCK regulatory segment. Moreover, Krueger *et al.* (*21*) recently used small-angle X-ray scattering and neutron scattering to observe directly CaM complexed with an enzymatically active truncation mutant of skeletal muscle MLCK. From that study, it appears that CaM undergoes an unhindered conformational collapse upon binding to MLCK, which activates the enzyme by inducing significant movement of the kinase's CaM-binding and autoinhibitory sequences away from the surface of the catalytic core. However, our understanding of the molecular mechanisms underlying enzyme activation by Ca^{2+}-CaM remains incomplete. As yet there has been little or no structural analysis of the sites where CaM interacts with functional MLCK, and the three-dimensional relationships between the catalytic core, the CaM-binding domain, and the autoinhibitory sites remain obscure.

Vi is a well characterized phosphate analogue, which binds to large number of phosphotransferases and phospho-

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Abbreviations: MLCK, myosin light chain kinase; MLC, myosin light chain; Vi, orthovanadate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; CaM, calmodulin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

hydrolases. Gibbons *et al.* (*22*) first reported that UV irradiation of dynein ATPase-bound Vi caused specific polypeptide cleavage. Since then, photomodification or photocleavage by irradiation has been used to study a variety of other enzymes including ribulose bisphosphate carboxylase/oxygenase (*23*), phosphofructokinase (*24*), Ca^{2+}-ATPase (*25, 26*), aldolase (*27*), and tubulin (*28*).

The chemical reactions involved in UV irradiation-induced polypeptide cleavage have been most extensively studied in myosin and adenylate kinase. With regard to myosin, UV irradiation of myosin/MgADP/Vi complexes caused oxidation of the hydroxymethyl side chain at serine 180, located in the myosin active site, to a serine aldehyde; further irradiation cleaved the myosin polypeptide at the affected serine residue (*29*). The precise molecular mechanism of the cleavage has been difficult to determine because the amino terminus produced by the cleavage has proved resistant to sequencing by Edman degradation. Nevertheless, the structure of the termini and a possible molecular mechanism of the cleavage at the serine residue were recently identified by Grammer *et al.* (*30*). In contrast to myosin, Vi photooxidation of adenylate kinase caused the peptide to be cleaved at proline 17 rather than at a serine residue (*31*). The mechanism of cleavage at the proline remains unclear.

In the present study, we utilized the Vi photooxidative cleavage reaction to study the interaction between the autoinhibitory region and the catalytic site of MLCK and to further characterize the regulation of MLCK activity by Ca^{2+}-CaM.

MATERIALS AND METHODS

Isolation of Proteins—Smooth muscle MLCK was purified from frozen turkey or chicken gizzards as previously described (*12*). CaM was prepared from bovine brain according to the methods of Walsh *et al.* (*32*). The 20-kDa smooth muscle myosin light chain (MLC) was isolated from gizzard myosin as described by Ikebe *et al.* (*33*).

Chemicals—Stock solutions of Vi (sodium ortho Vi, Aldrich) were prepared as described by Goodno (*34*).

Enzymatic Activity—Assays of MLCK activity were carried out using isolated 20 kDa light chain (0.1 mg/ml) in solution containing: 30 mM Tris-HCl, 1 mM $MgCl_2$, 50 mM NaCl, 0.2 mM $CaCl_2$, 5 μg/ml CaM, and 0.5 mM [γ-^{32}P]-ATP at pH 7.5. The amount of phosphorylation was estimated according to Walsh *et al.* (*32*).

SDS-PAGE—Electrophoresis was performed in 7.5-20% polyacrylamide gradient slab gels or 10% polyacrylamide slab gels in the presence of 0.1% SDS at a constant voltage (200 V) in the discontinuous buffer system of Laemmli (*35*). Apparent molecular masses were estimated using known molecular mass markers of smooth muscle myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), MLC, and α-lactalbumin (14.2 kDa).

Western Blotting and Immunostaining—Electrophoretic transfer of the polypeptide patterns from SDS-polyacrylamide gel to a nitrocellulose sheet was conducted over a period of 3 h at 6 V/cm by the method of Towbin *et al.* (*36*). The sheet was then cut into vertical strips, and one strip was stained with Amido-Black. The remaining strips were initially soaked in PBS-Tween buffer (PBS with 0.05% Tween 20) containing 2% bovine serum albumin for 2 h at room temperature in order to saturate nonspecific protein-binding sites. They were then placed in PBS-Tween buffer containing 10-100 μg/ml purified monoclonal antibodies against C- or N-termini for 1 h at room temperature. After a series of washings, the strips were soaked in PBS-Tween buffer containing 20 μg/ml peroxidase-conjugated anti-mouse IgG antibody for 30 min. The strips were washed again, and color was developed for 10 min in a fresh solution of 0.5% 4-chloro-1-naphthol, 50 mM Tris-HCl, 200 mM NaCl, and 0.02% H_2O_2 at pH 7.5. Finally, the strips were washed with water to stop the reaction and air-dried.

UV Irradiation—MLCK-containing solutions were placed on ice and irradiated with 366 nm UV light for selected periods of time using a UVL-56 lamp (16 W, Ultra-Violet Products) mounted 2 cm above the surface of the sample solutions.

Amino Acid Analysis of the 28 kDa Fragment of MLCK—The 28-kDa fragment generated from MLCK by Vi photooxidization cleavage was isolated by SDS-PAGE using 7.5-20% polyacrylamide gradient slab gels. The portion of the gel containing the 28-kDa fragment was excised, and the fragment was electrophoretically eluted from the gel according to the method of Hanaoka *et al.* (*37*). After hydrolysis of the isolated fragment in 6 N HCl, the sample was dried, derivatized with phenyl isothiocyanate, and analyzed using a PICO-TAG system (Waters). Sequence analysis was then performed on an ABI 473A protein sequencer (Applied Biosystems).

RESULTS

Effect of Vi on MLCK Activity in the Absence and Presence of ADP—To test whether Vi binds to the catalytic site of MLCK, we studied the effect of Vi on MLCK activity, which was assayed by measuring phosphorylation of MLC in the presence and absence of ADP. ADP is known to inhibit kinase activity of MLCK competitively. As shown in Fig. 1, about 30% inhibition was observed in the presence of 1 mM ADP, in agreement with the report by Ikebe and Hartshorne (*51*). In the presence of 1 mM ADP+1 mM Vi (1 mM ADP/Vi), MLCK activity was substantially inhibited, and phosphorylation of MLC was reduced to approximately one-third of control after 5 min (Fig. 1). In contrast, 1 mM Vi had no effect on MLCK activity when applied by itself (Fig. 1, filled circles). Figure 2 shows a double reciprocal plot of the MLCK activity *vs.* [ATP] at 0.4 mM and 0.8 mM ADP/Vi. ADP/Vi competitively inhibited ATP binding to MLCK, as reflected by the inhibition of kinase activity; the K_i for ADP/Vi was calculated to be 58 μM, while the K_m for ATP was 21 μM. These results suggest that Vi binds to the catalytic site of MLCK, and that when ADP is also bound, an ADP-Vi complex is formed that behaves as an ATP analogue with Vi occupying the γ-phosphate-binding site.

Effect of UV Irradiation in the Presence of ADP/Vi on the MLCK Activity—Earlier studies showed that UV irradiation of some enzymes in the presence of Vi elicited photomodification or cleavage of the polypeptide at or near the site of Vi binding (*22*). Moreover, experiments with skeletal muscle myosin indicated that UV irradiation-in-

duced reduction of MLCK activity may be due to side chain photooxidation of a serine residue located within the active site (*29*). We observed that irradiation of MLCK with 366-nm UV light in the presence of 1 mM ADP/Vi inhibited kinase activity in a time-dependent manner, and that after 25 min of irradiation, MLCK activity was reduced by 70-80% when normalized against the control (Fig. 3, open triangles). Addition of 1 mM ATP was partially protective, presumably as a consequence of competitive antagonism of ADP/Vi binding, and attenuating the inhibitory effect of ADP/Vi by approximately 25% after 25 min of irradiation (Fig. 3, filled triangles). Although some inhibition was observed, irradiation of MLCK in the presence of Vi alone was much less effective than ADP/Vi at reducing kinase activity (Fig. 3, filled circles), and in the absence of ADP and Vi, UV irradiation no effect on kinase activity (Fig. 3, open circles).

We reasoned that if the reduced kinase activity resulted from photooxidation of a serine, perhaps enzyme activity would be recovered when the side chain is again reduced (*29*). However, exposing MLCK to the reducing agent NaB^3H_4 following UV irradiation in the presence of ADP/Vi did not reestablish kinase activity, and no ^3H was incorporated into MLCK. This finding is inconsistent with the conclusion that photooxidation of a serine side chain is responsible for inactivating MLCK activity. Rather, photomodification of MLCK probably occurred on a different amino acid residue within the active site.

UV Irradiation-Induced Cleavage of MLCK in the Presence of ADP/Vi—The Vi-binding site was studied further by UV-irradiating MLCK and then analyzing the

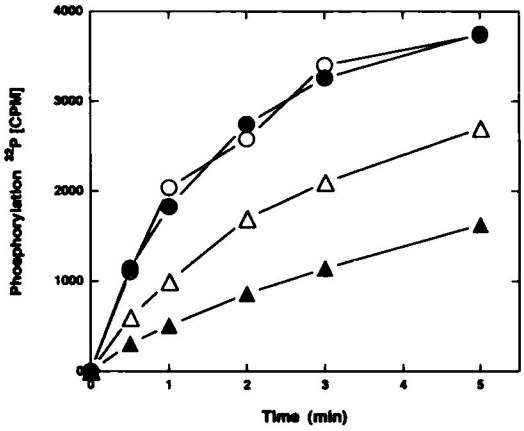

Fig. 1. **Time course of MLC phosphorylation by MLCK in the presence of ADP/Vi.** Conditions: 1 μg MLCK was incubated with 0.1 mg/ml MLC in a solution of 0.2 mM [γ-^{32}P]ATP, 50 mM NaCl, 30 mM Tris-HCl, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 5 μg/ml CaM at a pH 7.5 and 25°C (○) or in the presence of 1 mM Vi (●), or 1 mM ADP (△), or 1 mM ADP/Vi (▲).

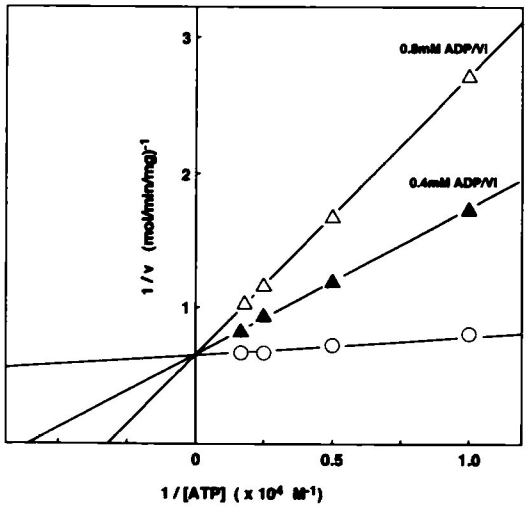

Fig. 2. **Double reciprocal plot illustrating competitive inhibition of MLCK activity by ADP/Vi.** MLCK activity was assayed under control conditions (○) and in the presence of ADP/Vi [0.4 mM/0.4 mM (▲) and 0.8 mM/0.8 mM (△)] in the dark. Conditions: 1 μg/ml MLCK was incubated with 0.1 mg/ml MLC in a solution of 5 μg/ml CaM, 50 mM NaCl, 30 mM Tris-HCl, 1 mM $MgCl_2$, and 0.2 mM $CaCl_2$, at pH 7.5 and in the presence of 50, 100, 200, or 400 μM [γ-^{32}P]ATP. Reactions were allowed to proceed for 1 min at 25°C and stopped with 5% trichloro acetic acid.

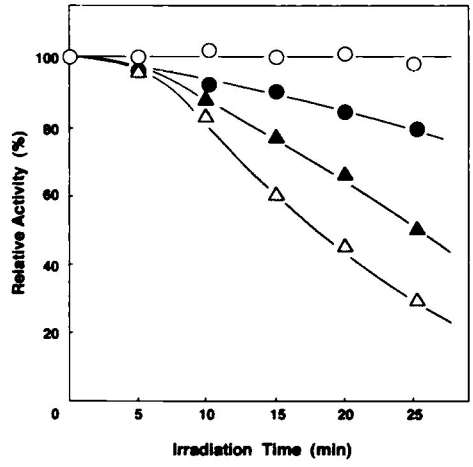

Fig. 3. **Photoinactivation of MLCK activity expressed as a function of irradiation time.** UV irradiation (366 nm) was carried out in the absence of Vi (○) or in the presence of 1 mM Vi (●), 1 mM ADP/Vi (△), or 1 mM ADP/Vi+2 mM ATP (▲) at 0°C for 0-20 min in a solution of 0.1 M NaCl, 50 mM MOPS, 1 mM $MgCl_2$, and 0.15 mg/ml MLCK.

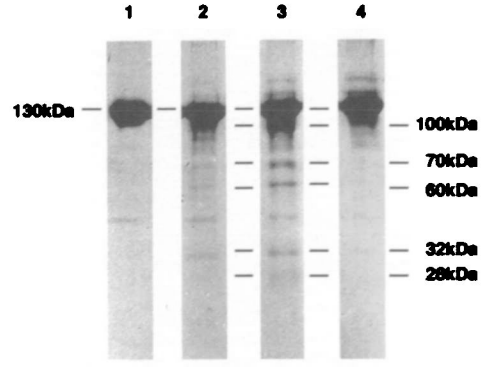

Fig. 4. **Vi-mediated photocleavage of MLCK shown by SDS-PAGE.** MLCK (0.3 mg/ml) was UV irradiated under control conditions (lane 1) or in the presence of 1 mM Vi (lane 2), 1 mM ADP/Vi (lane 3), or 1 mM ADP/Vi+2 mM ATP (lane 4). UV (366 nm) irradiation was carried out for 20 min at 0°C. Cleaved fragments were separated by SDS-PAGE on 7.5-20% acrylamide gradient gels.

samples using SDS-PAGE (Fig. 4). When MLCK was irradiated in the absence of ADP and Vi, the gels revealed no additional bands, indicating no MLCK cleavage (Fig. 4, lane 1). In contrast, UV irradiation for 15 min in the presence of 1 mM ADP/Vi resulted in cleavage of the 130-kDa MLCK polypeptide to produce new bands at 100, 70, 60, 32, and 28 kDa (Fig. 4, lane 3). The amount of MLCK cleaved increased to a maximum of 15-20% of the total sample after 30 min of irradiation in the presence of 1 mM ADP/Vi. Thus, while irradiation almost completely blocked kinase activity (Fig. 3), only a fraction of that inhibition can be attributed to MLCK cleavage. As expected, 2 mM ATP provided significant protection from cleavage (Fig. 4, compare lanes 3 and 4). UV irradiation in the presence of 1 mM Vi, alone also resulted in MLCK cleavage, but substantially less than was observed in the presence of ADP/Vi (Fig. 4, compare lanes 2 and 3). Cremo *et al.* (*50*) previously demonstrated that Vi readily forms oligomers easily at neutral pH which can bind to the ATPase site of myosin and induce Vi-mediated photocleavage. Therefore, the cleavage on MLCK in the presence of Vi alone may be due to binding of tetramer of Vi.

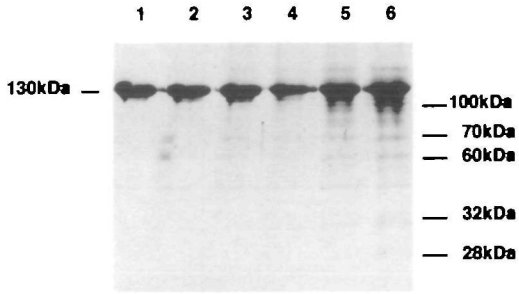

Fig. 5. **Effect of time of UV irradiation on Vi-mediated photocleavage of MLCK.** MLCK (0.3 mg/ml) was UV irradiated for 0, 2, 5, 10, 15, and 20 min (lanes 1-6, respectively) in the presence of 1 mM Vi and 1 mM ADP in a solution of 0.1 M NaCl and 50 mM MOPS. The irradiated samples were analyzed by SDS-PAGE on 7.5-20% acrylamide gradient gels.

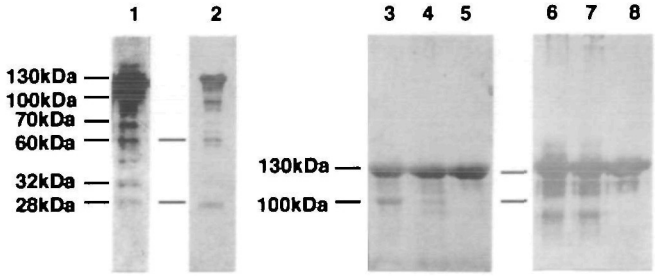

Fig. 6. **Identification of photocleaved fragments of MLCK using monoclonal antibodies.** MLCK was photocleaved in the presence of 2 mM ADP/Vi (lanes 1, 2, 3, and 6) or 2 mM Vi (lanes 4 and 7) for 20 min at 0°C. Cleaved fragments were separated by SDS-PAGE on 7.5-20% acrylamide gradient gel (lanes 1 and 2). To separate the 130- and 100-kDa fragments completely and to see only this region, 10% acrylamide iso gel was used (lanes 3-8), and proteins were transferred to nitrocellulose membranes and immunostained with antibodies recognizing the C-terminus of MLCK. Lane 1, protein staining of the SDS-polyacrylamide gel; lanes 2, 6, 7, and 8, immunostaining; lanes 3, 4, and 5, amide black staining; lanes 5 and 8, intact MLCK.

Increasing [Vi] to 4 mM or greater elicited non-specific cleavages that were not inhibited by addition of ATP (data not shown). As shown in Fig. 5, the Vi-mediated photocleavages were dependent on time of UV irradiation, and the 70- and 60-kDa fragments were generated earlier than other fragments.

The positions where MLCK was cleaved relative to the C-terminus were identified by labeling fragments containing the C-terminus with monoclonal antibodies specifically recognizing the C-terminus of MLCK. This was accomplished by irradiating samples in the presence of ADP/Vi, subjecting them to SDS-PAGE, and electroblotting them on nitrocellulose membranes. The membranes were then immunostained with C-terminus antibodies. As shown in Fig. 6, C-terminus antibodies labeled the 60- and 28-kDa fragments (Fig. 6, lane 2), but not the 70, 32, or 100 kDa (Fig. 6, lanes 2 and 6). Conversely, monoclonal antibodies recognizing the N-terminus of MLCK labeled only the 100-

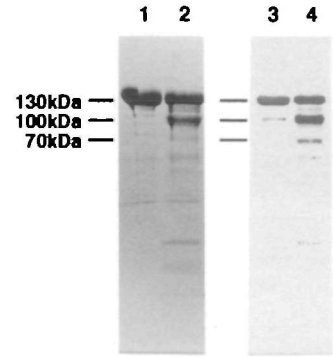

Fig. 7. **Identification of photocleaved fragments of MLCK using monoclonal antibodies recognizing the N-terminus of MLCK.** MLCK was irradiated with 366-nm UV in the absence (lanes 1 and 3) or in the presence of 2 mM ADP/Vi (lanes 2 and 4) for 25 min at 0°C. Cleaved fragments were separated by SDS-PAGE on 7.5-20% acrylamide gradient gels, transferred to the nitrocellulose membranes, and immunostained with antibodies recognizing the N-terminus of MLCK. Lanes 1 and 2, protein staining; lanes 3 and 4, immunostaining.

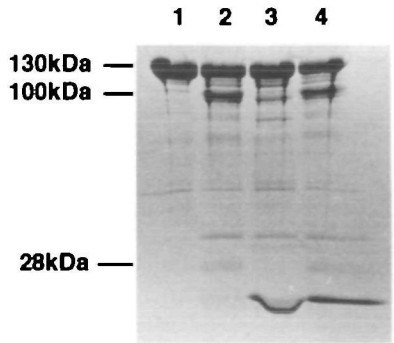

Fig. 8. **Effect of CaM on photocleavage of MLCK.** MLCK (0.3 mg/ml) was UV-irradiated for 15 min at 0°C in a solution of 120 mM NaCl, 30 mM MOPS, and 1 mM $MgCl_2$ (lane 1) or additionally in the presence of 2 mM ADP/Vi (lane 2); 2 mM ADP/Vi + 0.1 mg/ml CaM and 1 mM $CaCl_2$ (lane 3); or 2 mM ADP/Vi, 0.1 mg/ml CaM, and 2 mM EGTA (lane 4). Cleaved fragments were separated by SDS-PAGE on 7.5-20% acrylamide gradient gels.

and 70-kDa fragments (Fig. 7). Therefore, it appears that UV irradiation cleaves MLCK at two sites at 28 and 60 kDa from the C-terminus. Furthermore, the 100- and 70-kDa fragments are probably the remaining N-terminus fragments cleaved from the 28- and 60-kDa fragments, respectively. The 32-kDa fragment was not identified by N and C terminus antibodies. This fragment may be generated by cleavage of the 60-kDa fragment at the site at 28 kDa from the C terminus of MLCK.

Calcium-Calmodulin Effect on the Photocleavage of MLCK—Application of Ca^{2+}-CaM substantially inhibited UV-induced photocleavage, resulting in formation of the 100- and 28-kDa fragments, although the 60- and 70-kDa fragments continued to be produced (Fig. 8, lane 3). Thus, exposure to Ca^{2+}-CaM selectively protects against cleavage in the region 28 kDa from the C-terminus, which includes the CaM-binding site and autoinhibitory site. In addition, blockade of photocleavage by Ca^{2+}-CaM was Ca^{2+}-dependent, indicating that cleavage was antagonized upon CaM binding to MLCK. In the absence of Ca^{2+}, CaM is not bound to MLCK, and no effect of CaM on Vi-mediated generation of 100- and 28-kDa fragments was observed (Fig. 8, compare lanes 2 and 4).

The data presented so far show that ADP/Vi competitively inhibited MLCK activity (Fig. 2) and that photocleavage was completely blocked by the addition of ATP (Fig. 4). This means that Vi-mediated photocleavage must occur at an amino acid lying within the catalytic site, and it suggests that inhibition of photocleavage by Ca^{2+}-CaM results from a conformational change that displaces the regulatory site away from the catalytic site.

Amino Acid Analysis of the 28 kDa Fragment—To determine at which amino acid MLCK was cleaved, the 28-kDa fragment was isolated, and the N-terminal sequence was analyzed by Edman degradation using a gas phase sequencer. However, the 28-kDa fragment proved resistant to sequencing, indicating that cleavage did not generate a free α-amino group at the N-terminus. Nevertheless, we were able to estimate the location of the cleavage site within the regulatory domain using the method of Nascimento *et al.* (*38*), which is based on a comparison of the amino acid composition of the purified 28-kDa fragment with the amino acid sequence of MLCK determined from its cDNA (*5*). The molar ratios of the individual amino acids in the entire 28-kDa fragment were calculated from the pmol values obtained by amino acid analysis. They were compared with molar ratios of theoretical MLCK fragments whose amino acid composition and molecular weights were calculated from the known primary sequence. For example, the values expressed in Table I (Analysis) are based on the apparent molecular mass of the 28-kDa fragment and a theoretical cleavage site 802 amino acids from the C-terminal, which gives a molecular weight of 15,549. The exact molar ratios of the amino acids determined from the known sequence are shown in Table I (Sequence). The differences between the two calculated molar ratios for each amino acid are expressed as a percent of the value calculated from the known sequence and shown Table I (Difference).

We surmised that the sums of the differences between the molar ratios calculated from amino acid analysis and from the known sequence (f^*; Σanalysis−sequence) would be minimized when the theoretical cleavage was assumed to occur at or near the site of the actual irradiation-induced cleavage. The results obtained for several fragments

TABLE I. **Analysis of the amino acid composition of purified MLCK 28-kDa fragment photocleaved in the presence of ADP/Vi using molar ratios obtained from the known amino acid sequence of MLCK.**

Amino acid	(pmol)	Analysis[a] (mol/mol)	Sequence[b] (mol/mol)	Difference[c] (%)
Asx	26.75	18.4	17	8.3
Glx	44.32	30.8	31	0.7
Ser	20.47	14.1	13	8.2
Gly	23.33	16.1	15	7.5
His	4.23	2.8	3	5.3
Arg	8.31	4.2	5	16.0
Thr	13.48	9.3	9	3.2
Ala	18.07	12.4	13	4.3
Pro	15.17	10.4	7	49.1
Tyr	6.48	4.5	5	10.7
Val	16.78	11.5	11	4.9
Met	8.71	6.0	6	0.0
Cys	–	5[d]	5	0.0
Ile	11.92	8.2	7	17.2
Leu	19.63	13.1	7	87.1
Phe	6.52	4.5	4	12.5
Lys	14.72	10.1	11	8.2
Trp	–	1[d]	1	0.0
f^*: Σanalysis−sequence (%) = 243.2				

[a]The molar ratios of the individual amino acids in the 28-kDa MLCK fragment were calculated from pmol values obtained by amino acid analysis of the polypeptide, as described in "MATERIALS AND METHODS." The molecular weight value for the 28-kDa fragment was calculated from the known primary sequence of an arbitrarily selected theoretical cleavage site. The values expressed in the table were calculated based on the MLCK fragment having an apparent molecular mass of 28 kDa and a theoretical cleavage site 802 amino acids from the C-terminus, which gives a molecular weight of 18,531. [b]Once a theoretical cleavage site is selected, the exact molar ratio of the individual amino acids is determined from the known sequence. [c]The difference between the molar ratios calculated from amino acid analysis and the exact molar ratios based on the sequence is expressed as a percentage of the exact molar ratio. [d]Cys and Trp levels were not determined by amino acid analysis. Therefore, values were introduced into the calculation based on the sequence data.

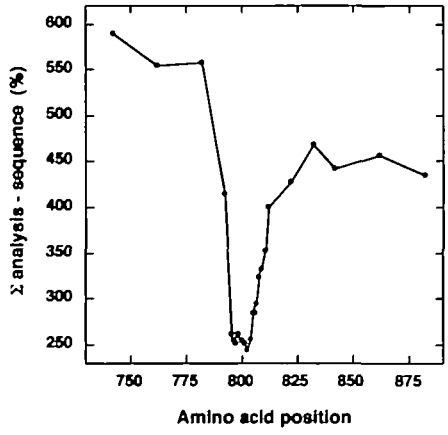

Fig. 9. **Estimation of the photocleavage site on MLCK that generates the 28-kDa polypeptide.** The sum of the differences between molar ratios calculated based on amino acid analysis *versus* molar ratios calculated from the known sequence (f^*: Σanalysis−sequence) were determined for several theoretical amino acid positions as described in Table I and plotted as a function of that position.

cleaved at various amino acid positions are shown in Fig. 9. This analysis produced a clear minimum at Lys 799±3 amino acids. Interestingly, this means that site of UV irradiation-induced cleavage lies well within the MLCK pseudosubstrate region.

DISCUSSION

Vi is a phosphate analog known to interact with a variety of phosphotransferases and phosphohydrolases, forming reaction intermediates within the active site and resulting in inhibition of enzymatic activity. Here, we report for the first time that Vi can be utilized as a probe for analyzing the structure-function relationship of protein kinases.

MLCK activity was inhibited by ADP/Vi, which was competitive with ATP. Furthermore, the inhibition required the presence of ADP. These results suggest that Vi is incorporated into the active site. It is known of myosin ATPase that ADP/Vi tightly binds to the active site of myosin to form a stable ternary complex of myosin/ADP/Vi (*39*). In contrast, while ADP/Vi can inhibit MLCK activity in a competitive manner with ATP, both ADP and Vi were readily separated from MLCK by gel filtration, suggesting that a stable ternary complex is not formed for MLCK.

While UV irradiation in the presence of 1 mM ADP/Vi inhibited MLCK activity by more than 70%, the peptide cleavage occurred in a maximum of only 15% of the total irradiated MLCK. This discrepancy between the photocleavage and the inhibition in the kinase activity suggests that the process of photocleavage proceeds in two steps, as suggested earlier for the Vi-induced photocleavage of myosin (*40*). First, oxidation of an amino acid residue near the bound Vi results in the inhibition of the kinase activity; and second, the cleavage takes place at the oxidized amino acid residue by an unknown mechanism. Since Vi is a phosphate analog and ADP/Vi induced photocleavage was completely blocked by the addition of ATP (Fig. 4), Vi-mediated photocleavage must occur at an amino acid near the phosphate-binding site in the active site pocket. At least two amino acid residues are photo-oxidized, since MLCK polypeptide is cleaved at two sites. One of them is identified as Lys 799±3 residues, which lies within the autoinhibitory region and calmodulin-binding site. Consistent with this, the inhibition of photocleavage at this site is blocked by Ca^{2+}-CaM. There are two possibilities to account for the Ca^{2+}/CaM-dependent inhibition of photocleavage. First, it is known that Trp 800 is one of the most critical CaM-anchoring sites. Therefore, CaM binding, *i.e.*, the interaction of Trp 800 with the hydrophobic pocket of CaM, might change the chemical reactivity of the neighboring residues to Vi. Second, the CaM binding may dissociate the regulatory region from the Vi-binding site, *i.e.*, ATP-binding site, as suggested earlier (*10*).

To determine the exact cleavage site in the regulatory region, we attempted to analyze the amino acid sequence of the 28-kDa fragment using Edman degradation. However, we were unable to determine the sequence, presumably because the fragment lacks a free N-terminus. Likewise, we were unable to sequence the 60-kDa fragment. Similar results have been reported by other investigators (*28, 31, 41, 43*). Recently, Nascimento *et al.* (*38*) succeeded in estimating the calpain-cleavage site of myosin V by comparing the amino acid composition of the produced fragment with the known sequence of myosin V. Since this fragment contained a free N-terminus, they were able to confirm the cleavage site by direct sequencing analysis and found that the site estimated by amino acid composition analysis lay within two amino acid residues from the actual cleavage site. This method is thus quite reliable. In this study, we employed this method to estimate the Vi induced cleavage site of MLCK as Lys 799±3 amino acid residues. Interestingly, this site lies near the center of the autoinhibitory/calmodulin-binding region of MLCK (Fig. 10).

It has been shown for myosin that Vi-induced photo-oxidation-mediated peptide bond cleavage occurs at serine (*29*) and proline (*31*). However, the absence of serine and proline residues near the Vi-induced cleavage site of MLCK (Lys 799±3 residues) suggests that Vi-induced photooxidation occurs at other amino acid residues. The oxidation of an -OH group seems to be an important step for the subsequent peptide bond cleavage (*42*), and it has been suggested that in addition to a serine residue, the oxidation of a threonine residue also causes the peptide bond cleavage (*45*). We showed previously that Thr 802 can be autophosphorylated through an intramolecular process (*46*), suggesting that Thr 802 is in close contact with the γ-phosphate of ATP bound to the active site of MLCK. Therefore, it is plausible that Thr 802 is the residue photo-oxidized in the presence of ADP/Vi.

In the presence of Ca^{2+}-CaM, photocleavage in the region of 28 kDa from the C-terminus was largely prevented, and

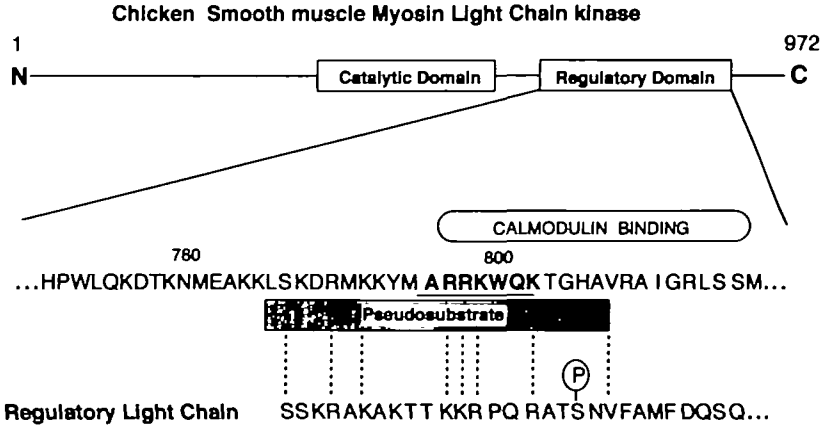

Fig. 10. **Schematic representation illustrating the likely Vi-mediated photocleavage site in the regulatory domain of chicken smooth muscle MLCK.** The line above the pseudosubstrate sequence indicates the possible cleavage site estimated from the analysis of the amino acid composition of the 28-kDa fragment.

this resulted in the disappearance of the 100- and 28-kDa bands in the SDS-PAGE (Fig. 8). The results agree with the finding that the cleavage site of the 100- and 28-kDa fragments is in the autoinhibitory/calmodulin-binding region. In contrast, formation of the 60- and 70-kDa fragments was not affected by the addition of Ca^{2+}-CaM and MLC. Because of the limited quantity of the 60-kDa fragment produced and its larger molecular mass, we were unable to determine the cleavage site of the 60- and 70-kDa fragments accurately. However, it should be noted that photo-cleavage near the ATP-binding consensus motif, *i.e.*, GXGXXG, would produce fragments with similar apparent molecular masses. Previously, Cremo *et al.* (*29*) showed that Vi-induced photo-cleavage occurs at the serine residue in the ATP-binding consensus sequence of myosin. The ATP-binding motif of MLCK (GSGKFG) contains one serine residue, which is thus likely to be the photo-cleavage site producing the 60- and 70-kDa fragments.

Since Vi-induced photocleavage of the 60- and 70-kDa fragments was unaffected by calmodulin binding, the results also suggest that calmodulin binding does not change the Vi binding to the γ-phosphate-binding site, in particular, the interaction between Vi and the residues involved in the formation of the γ-phosphate-binding pocket. Previously, it was found that calmodulin binding to MLCK does not markedly change the affinity labeling of MLCK with the ATP analog to the active site (*47*). The present results are consistent with this earlier finding and suggest that calmodulin binding does not alter the active site conformation.

It has been suggested that the regulatory region of MLCK folds back on the catalytic region, resulting in inhibition of kinase activity (*16*). Evidence obtained using synthetic peptides (*9, 12*), truncation of MLCK by proteolysis (*12, 44*), and mutagenetic analysis of MLCK (*14, 15, 48, 49*) has supported this hypothesis. The inhibitory function of the regulatory domain was assigned to a sequence near the N-terminal of the calmodulin-anchoring site (*9*), and because of the sequence similarity of this region to the substrate, *i.e.*, myosin regulatory light chain, the autoinhibitory region was termed a "pseudosubstrate" inhibitor sequence (*9, 16*). This view has been questioned, since the deletion and/or substitution of the pseudosubstrate residues failed to de-inhibit MLCK activity (*48, 49*), but Tyr 794 lying in the middle of the autoinhibitory region plays a critical role in autoinhibition of the kinase, suggesting that the autoinhibitory segment around Tyr 794 is in close proximity to the catalytic site (*49*). The present study suggests that the autoinhibitory segment lies sufficiently close to the γ-phosphate-binding site in the active site to be oxidized by Vi bound within the catalytic site, presumably at the γ-phosphate-binding site (Fig. 11). This is the first biochemical evidence that the three-dimensional structure of MLCK brings the regulatory site into such close contact with the catalytic site.

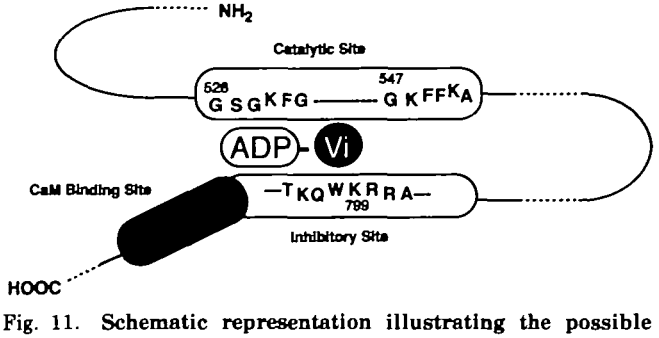

Fig. 11. **Schematic representation illustrating the possible direct interaction of the MLCK regulatory domain with the ATP-binding site.**

In the present study, we have employed Vi-mediated photocleavage methods to study the regulatory mechanism of MLCK activity by Ca^{2+}-CaM. The results suggest that the three dimensional structure of MLCK brings the autoinhibitory region into direct contact with the catalytic site and that the binding of Ca^{2+}-CaM induces a conformational change, displacing the autoinhibitory region away from the catalytic site.

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