Modulation of Smooth Muscle Myosin Light Chain Kinase Activity by Ca^{2+}/Calmodulin-Dependent, Oligomeric-Type Modifications†

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ABSTRACT: Oligomerization of turkey gizzard myosin light chain kinase (MLCKase) was demonstrated by a zero-length cross-linker, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC), a standard reagent used in investigations of specific protein−protein interaction [Mornet et al. (1989) *J. Muscle Res. Cell Motil. 10,* 10−24]. This approach revealed that in solution the kinase was not monomeric but the monomers were in equilibrium with the kinase dimeric and oligomeric forms. Addition of Ca^{2+}/calmodulin (CM) shifted this equilibrium in the direction of the kinase dimers, accompanied by a 2-fold decrease of the kinase catalytic activity, in addition to a 2-fold decrease of its apparent affinity for CM [Sobieszek et al. (1993) *Biochem. J. 295,* 405−411]. The dimer (and/or oligomer) formation was shown to result from an interaction of the kinase autoinhibitory domain with its 24 kDa tryptic fragment containing titin-like domain II-3. The possible significance of the oligomerization in regulation of MLCKase activity is discussed.

Myosin light chain kinase (MLCKase)[1] is a calmodulin (CM)-dependent enzyme responsible for the regulation of actin−myosin interaction in smooth muscle [for a review, see Hartshorne (1987) and Stull et al. (1993)]. The kinase, being completely inactive in the absence of Ca^{2+}/CM, represents the primary molecular switch leading to contraction of this muscle type. An increase in myoplasmic Ca^{2+} concentration results in the formation of a Ca^{2+}_4/CM complex which binds to the kinase at 1:1 stoichiometry and activates the enzyme. The active enzyme phosphorylates the regulatory light chain of myosin, permitting a cyclic interaction of myosin heads along actin filaments.

According to a current hypothesis, MLCKase activity is regulated by a pseudosubstrate autoinhibitory domain, homologous to the phosphorylation site on the myosin regulatory light chain (LC_{20}), which blocks the active site in the absence of Ca^{2+} and/or CM (Kemp & Pearson, 1991). The autoinhibitory model appears to be widely accepted, not only for the MLCKase but also for several other CM-dependent enzymes such as the plasma membrane Ca pump (Vorherr et al., 1990), CM kinase II (Colbran et al., 1988), or calcineurin (Hashimoto et al., 1990), but the data collected so far are largely inferential. More recent observations on binding of mutant CMs by the kinase without its activation (VanBerkum & Means, 1991) indicate that besides the autoinhibition, an additional conformational change may be necessary for the enzyme to perform its function.

Our own recent data indicate that smooth muscle MLCKase may exhibit some allosteric properties which cannot be explained in terms of 1:1 MLCKase/CM binding stoichiometry. It has been shown that this kinase is activated by CM with a high degree of cooperativity (Sobieszek, 1991). The cooperativity results from Ca^{2+}/CM-dependent, possibly oligomeric modification of kinase, indicating a more complex regulation of enzyme by Ca^{2+} (Sobieszek at al., 1993). Among other Ca^{2+}/CM-dependent enzymes, only two may have a similarly complex regulatory mechanism. One of them is the classical allosteric enzyme phosphofructokinase (PFK), the properties of which were extensively studied [see Buschmeier et al. (1987) and Kurganov (1982)], but only recently a possible CM involvement in its regulation has been demonstrated (Mayr & Helmeyer, 1983; Mayr, 1984a,b). The other enzyme, erythrocyte Ca^{2+}-ATPase, is regulated by oligomerization as well as by Ca^{2+}/CM (Kosk-Kosicka et al., 1990).

In the present study, we have investigated the oligomeric properties of turkey gizzard MLCKase by application of a zero-length cross-linker together with SDS−PAGE. This approach provided a direct demonstration that the kinase readily forms dimers which are at equilibrium with kinase oligomeric and monomeric species. At the same time, we have characterized some kinetic properties of these oligomeric forms.

EXPERIMENTAL PROCEDURES

Myosin light chain kinase and its activator calmodulin were purified from turkey gizzard as previously described (Sobieszek et al., 1991). The myosin regulatory light chain of turkey gizzard myosin, used as MLCKase substrate, was isolated by the method of Perrie and Perry (1970) and purified as described previously (Sobieszek, 1988). Concentrations of the light chain were measured by the biuret method (Gornall at al., 1949), while those of MLCKase and

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[1] Abbreviations: EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; CM, calmodulin; MLCKase, smooth muscle myosin light chain kinase; LC_{20}, smooth muscle myosin regulatory light chain; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, dithiothreitol; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; sulfo-NHS, *N*-hydroxysulfosuccinimide.

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CM were measured from their absorption at 278 nm using the extinction coefficient $E^{0.1\%} = 1.1$ for the kinase (Adelstein & Klee, 1981) and $E^{0.1\%} = 0.18$ for CM (Klee, 1978) with the respective molecular masses of 107.5 and 16.7 kDa (Olson et al., 1990). 1-Ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride (EDC) was purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals used were of analytical grade and were purchased either from MERCK (Darmstadt, Germany) or from Fluka Chemie AG (Buchs, Switzerland).

Phosphorylation rate measurements were carried out at 25 °C in "AA" buffer with the following composition (mM): KCl, 60; $MgCl_2$, 2; DTE, 0.5; and imidazole, 10, with the pH adjusted to 7.5 at 4 °C. The concentrations of MLCKase, calmodulin, and $CaCl_2$ used in the assay were 100 nM, 120 nM, and 0.1 mM respectively. Saturated concentrations of the substrate (purified LC_{20}) were used, and these were in the range of 250−300 μM. For more details, see Sobieszek (1991).

Unless otherwise stated, cross-linkage of MLCKase (5−8 μM) by EDC (5 mM) was carried out at 25 °C for 30 min in the AA buffer containing additionally either 0.1 mM $CaCl_2$ or 1 mM EGTA. Control experiments demonstrated that the same cross-linkage patterns were obtained at 0 °C. CM, when present, was added in a molar concentration of 1.5 times above that of kinase. The cross-linkage was terminated by addition of 2-fold excess of DTE.

Dimerization and/or oligomerization of the kinase was analyzed by a mini gel SDS−PAGE system essentially as described by Matsudaira and Burgess (1978), using a 3% stacking gel and a gradient (9−20%) separating gel with modifications and improvements described in detail recently (Sobieszek, 1994b).

Tryptic hydrolysis of MLCKase (5 μM) was performed at 25 °C in the AA buffer, containing additionally 1 mM EGTA, with a kinase to trypsin ratio of 40:1 (w/w). When indicated, the EGTA was replaced by 0.1 mM $CaCl_2$. CM was added in a molar concentration of 1.5 times above that of kinase. The trypsinolysis was terminated by addition of soya bean inhibitor at 2-fold molar excess with respect to trypsin or by boiling of the samples after addition of an aliquot of an "SDS-mix" [final concentration: 3% SDS, 73 mM Tris-HCl (pH 6.8), 1.2% β-mercaptoethanol].

RESULTS

Effect of EDC on MLCKase Activity. EDC is one of the most commonly used cross-linkers in studies of the oligomeric properties of proteins. This water-soluble reagent forms zero-length covalent bonds between amino and carboxyl groups in the contact area between two protein molecules (Mornet et al., 1989). At the beginning, we established the effects of EDC and cross-linkage on the activity of MLCKase. As shown in Figure 1A, incubation of turkey gizzard MLCKase with 5 mM EDC in the absence of calmodulin resulted in a relatively small decrease in kinase activity, amounting to a 10% reduction in the initial 30 min. A similar small reduction was observed with CM but with Ca^{2+} removed by 2 mM EGTA. In the presence of Ca^{2+}/CM, the decrease was significantly greater and amounted to 30−40% during the same period (Figure 1B, open circles). However, for most of the kinase preparations, a similar reduction was also observed for the kinase incubated with

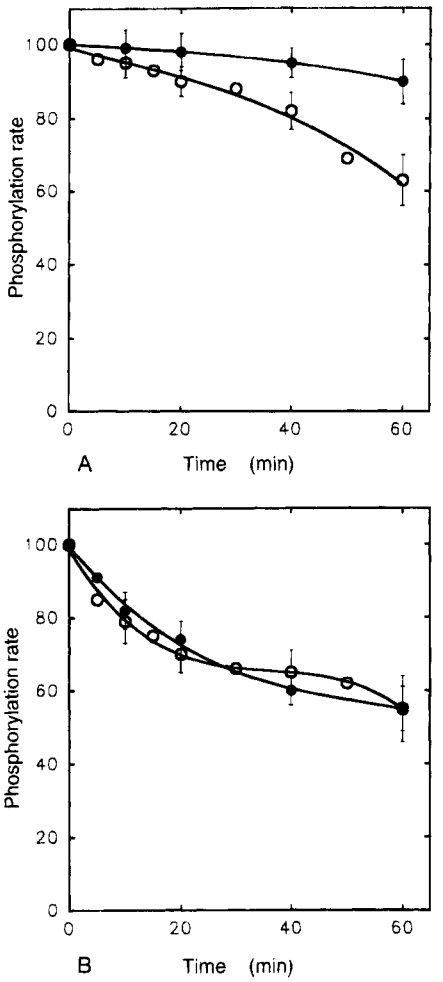


FIGURE 1: Effect of zero-length cross-linker (EDC) on MLCKase activity in the absence (A) and presence (B) of Ca^{2+}/CM. The kinase was preincubated with EDC (open symbols), and at given time points, aliquots were withdrawn for activity measurements. The controls (kinase preincubated in the absence of EDC) are shown by closed symbols. The measured rates ($n = 4-6$) were normalized at 100% equal to 660 ± 55 min^{-1}, representing the specific activity of the untreated kinase. Note that in the presence of Ca/CM (B), the EDC-treated and untreated kinases exhibited the same decay of the activity. For more details, see text.

CM in the absence of EDC (Figure 1B, closed circles); therefore, the reduction was not a consequence of the EDC treatment but resulted from a Ca^{2+}/CM-dependent inhibition of the type described earlier (Sobieszek et al., 1993), which was observed at substoichiometric ratios of CM to MLCKase. The relatively slow rate of the modifications observed here is a consequence of saturating concentrations of CM relative to the kinase.

MLCKase Oligomerization As Revealed by Cross-Linkage and SDS−PAGE. SDS−PAGE patterns of the EDC-treated kinase exhibited, in addition to the 125 kDa kinase monomer band, a strong band of mass 190 kDa, corresponding to cross-linked kinase dimer, and weaker bands with masses of approximately 300, 400, and 600 kDa representing its cross-linked trimeric, tetrameric, and hexameric forms (Figure 2). It has to be pointed out, however, that the EDC cross-linkage patterns revealed underestimated ratios of the oligomers/dimers to monomers, since the intermediate products of EDC reaction are subjected to hydrolysis, resulting in decreased coupling efficiencies (Staros et al., 1986). As shown in Figure 2B,C, the revealed extent of the kinase oligomer-

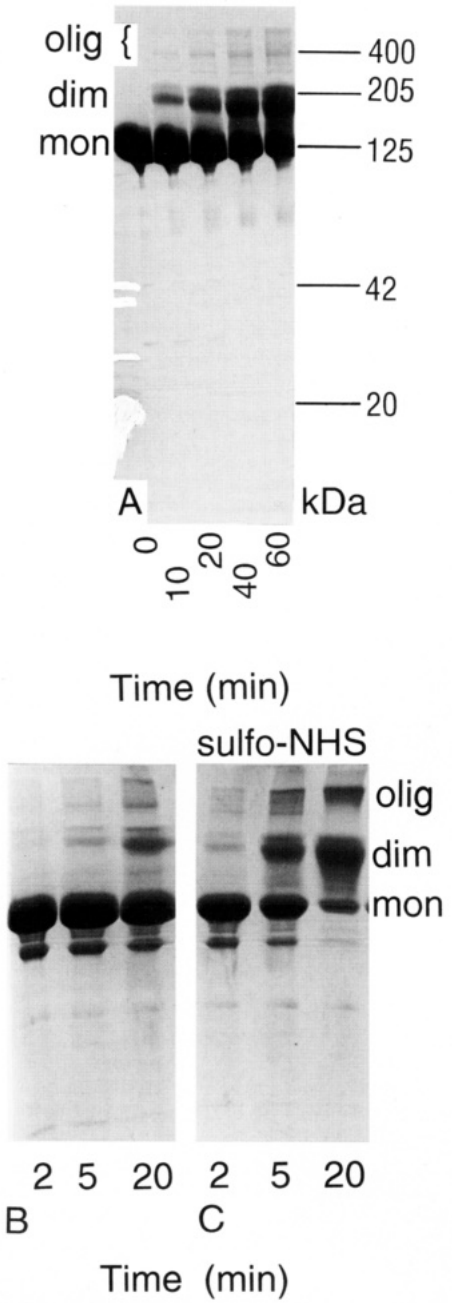
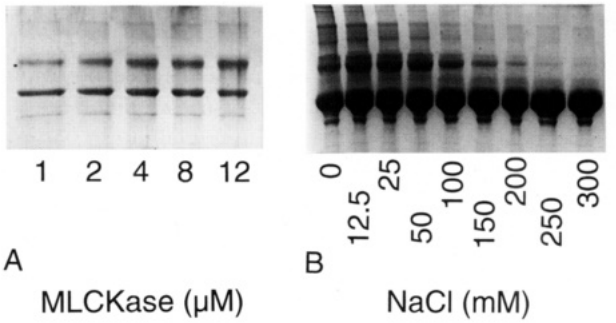


FIGURE 2: SDS−PAGE patterns of cross-linked MLCKase demonstrating its dimeric and oligomeric forms. The kinase was cross-linked by EDC under standard conditions (5 mM EDC) (A) or at 2.5 mM EDC with (C) or without (B) 1.25 mM sulfo-NHS. At the times indicated, aliquots were withdrawn and submitted to SDS−PAGE. Note the much higher cross-linkage efficiency in the presence of added sulfo-NHS.

ization was much higher if the cross-linkage was performed in the presence of *N*-hydroxysulfosuccinimide (sulfo-NHS). This reagent enhances the yield of water-soluble carbodiimide-mediated coupling by formation of more hydrolysis-resistant active intermediates (Staros et al., 1986).

The kinase cross-linkage or its oligomerization depended on the kinase concentration and the ionic condition of the medium. As expected for the oligomeric type of interaction, an increase in the MLCKase concentration from 1 μM to 12 μM resulted in a shift of the equilibrium in the direction of dimers and oligomers (Figure 3A). The dependence of the kinase oligomerization on NaCl concentration exhibited more complex character (Figure 3B). The relative concentrations



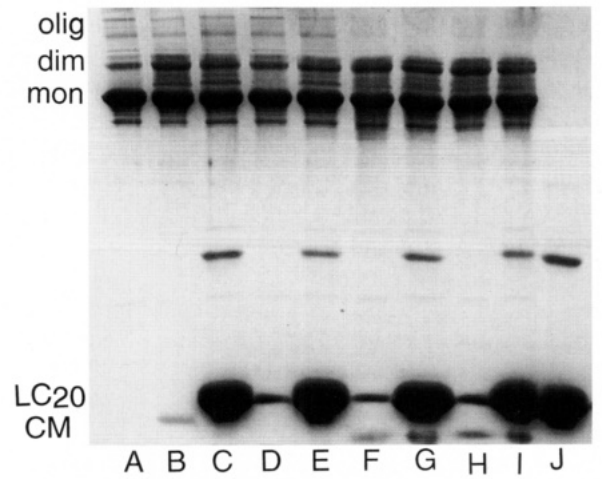


FIGURE 4: Cross-linkage of MLCKase in the presence of its substrates. A, MLCKase alone (control); B, MLCKase/CM/EGTA; C, MLCKase/LC_{20}; D, MLCKase/ATP; E, MLCKase/LC_{20}/ATP; F, MLCKase/CM; G, MLCKase/CM/LC_{20}; H, MLCKase/CM/ATP; I, MLCKase/CM/LC_{20}/ ATP; J, LC_{20}. In all cases, except (B), 0.1 mM $CaCl_2$ was present. The concentrations of MLCKase, CM, LC_{20}, and ATP were 8, 12, 100, and 300 μM, respectively. Note that the substrates [ATP and LC_{20}] did not affect the kinase oligomeric equilibrium. The loading for (A) was somewhat lower, and as a result, the control appears to have less dimers.

of cross-linked MLCKase oligomers decreased in parallel with increasing NaCl concentrations, indicating the ionic nature of oligomer formation. In contrast, at low NaCl concentrations, the relative concentration of the cross-linked dimers increased to a maximum at 12.5−25 mM NaCl and then rapidly decreased with increasing NaCl concentration.

As shown in Figure 4, the patterns of the cross-linked kinase were not significantly altered by addition of CM, provided Ca^{2+} was removed by EGTA (EGTA itself did not affect the cross-linkage pattern). Similarly, the kinase substrates (LC_{20} and ATP) did not influence the relative concentrations of the different kinase forms. However, SDS−PAGE patterns of the kinase cross-linked in the presence of Ca^{2+}/CM demonstrated an increase in the concentration of the kinase dimers relative to that of the monomers and oligomers. In this case, the bands corresponding to the kinase oligomers were essentially absent. In addition, we observed weaker bands in the range of 140−150 kDa corresponding to the kinase monomers cross-linked with CM and/or with LC_{20} (Figure 4).

Cross-linkage by EDC was also used to monitor the effects of Ca^{2+}/CM on the oligomerization of the kinase as a function of time in the presence or absence of 0.1 mM $CaCl_2$

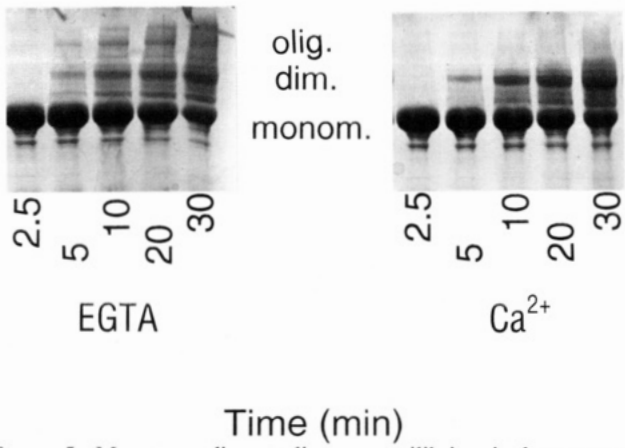


FIGURE 5: Monomer−dimer−oligomer equilibrium in the presence and absence of Ca^{2+}/CM. Note an increase in the relative concentration of the kinase dimers and a decrease in that of the oligomers in the presence of Ca^{2+}/CM.

(Figure 5). As before (see Figure 4), Ca^{2+}/CM shifted the equilibrium in the direction of kinase dimers, resulting in a reduction in the relative concentration of oligomers.

Partial Separation and Activities of the Kinase Oligomeric Forms. Measurements of the activities of native and cross-linked kinases as a function of CM concentrations demonstrated that the observed modification of the activity (see above) did not result from a reduction in the kinase affinity for CM but mainly from a reduction in its maximal rate (Figure 6A). This has been confirmed independently by application of the cross-linked kinase onto a CM-affinity column. As with the native enzyme, the cross-linked kinase only bound to the column in the presence of $CaCl_2$ and could be eluted in buffer containing EGTA (Figure 7A). A similar binding was observed for the kinase cross-linked in the presence of saturating concentrations of Ca^{2+}/CM (Figure 7B). Thus, the native kinase and also the EDC-treated monomeric and dimeric kinase forms bind CM with a similar high affinity. Furthermore, CM cross-linked with the kinase as well as free CM was also bound by the affinity gel. This indirect binding may take place only via dimerization and/or oligomerization of the kinase. This suggested that some kind of "sharing" of CM between the kinase molecules was taking place.

To obtain information on the properties of the different kinase forms (e.g., dimers and oligomers), we attempted to purify the forms cross-linked by EDC. In an initial approach, we applied the cross-linked MLCKase onto a Sephacryl S-200 gel-filtration column. The kinase eluted from such columns in a single peak containing all the activity, but some separation between the cross-linked oligomeric and dimeric species was apparent. The ascending fractions of the kinase peak were enriched in the oligomers whereas descending fractions contained mainly dimers and unmodified kinase (Figure 7C,D). Measurements of the activities of the fractions as a function of CM concentration demonstrated that the oligomers exhibited an increased affinity for CM compared with the dimers (see Discussion).

A better separation between the cross-linked and the intact kinase species was obtained by application of ion exchange chromatography. In this case, EDC−treated MLCKase eluted from DEAE-Sepharose column in two separate peaks. As shown in Figure 7E,F, the first DEAE peak contained mainly the cross-linked dimers while in the second predominantly unmodified kinase was present. A kinetic examination of the dimer fraction demonstrated that its initial rate was 2-fold lower compared to that of the native (unmodified) kinase (Figure 6B). Therefore, the activity decrease following EDC cross-linkage (or preincubation with CM) resulted from a reduction in the initial rate during kinase dimerization.

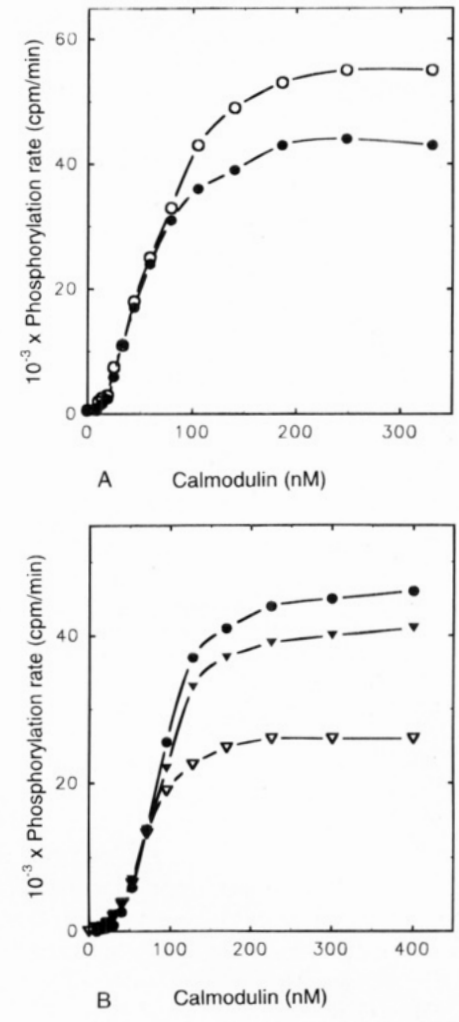


FIGURE 6: Activation of cross-linked and native MLCKases by calmodulin. In (A), the activation curves were obtained for native (open circles) and EDC-treated (closed circles) kinases without separation of its oligomeric forms. In (B), the activities of purified dimers (open triangles) and EDC-treated monomers (closed triangles) as well as the control (native kinase) are given. Note that at saturating concentrations of CM there was a 2-fold difference in the initial rates of the cross-linked kinase dimers and the unmodified kinase.

Domains Involved in Kinase Dimerization. In order to establish the kinase domain(s) involved in dimer and/or oligomer formation, SDS−PAGE patterns of the cross-linked kinase hydrolyzed with trypsin (Figure 8B) were compared to those of the hydrolyzed kinase subsequently cross-linked with EDC (Figure 8C,D). As shown in Figure 8A, the polypeptide pattern of the hydrolyzed kinase (in the presence of CM with Ca^{2+} removed by EGTA) was the same as previously reported (Ikebe at al., 1987) with four major fragments of 67, 64, 61, and 24 kDa. Minor fragments of 85, 80, and 36 kDa were also present, and a relatively abundant fragment of 28 kDa formed only after extensive trypsinolysis (a band of 17 kDa corresponded to CM added to the preparations). Trypsinolysis of the kinase in the presence of Ca^{2+}/CM resulted in formation of the same

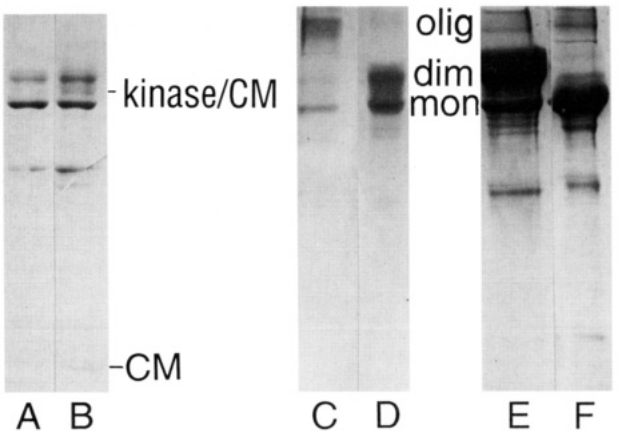


FIGURE 7: Separation of the oligomeric, dimeric, and monomeric forms of cross-linked kinase. (A) SDS−PAGE of the kinase cross-linked in the absence of Ca^{2+}/CM and eluted from the CM-affinity column by 2 mM EGTA. (B) The kinase was cross-linked in the presence of CM and eluted as in (A). Note that CM cross-linked to kinase as well as free CM was bound by the affinity gel. (C, D) "Ascending" and "descending" fractions (see text) of the kinase cross-linked as in (A) obtained from a Sephacryl S-300 gel-filtration column. (E, F) First and second peaks (see text) of the cross-linked kinase separated by gradient elution on a DEAE-Sepharose ion-exchange column.

fragments but only with transitory formation of the 64 kDa one; e.g., after 40 min trypsinolysis, there were only 61, 28, and 24 kDa fragments present (Figure 8A, line labeled "Ca^{2+}").

The trypsin degradation patterns of the cross-linked kinase were very similar (Figure 8B), except for the relative abundance of the 85 and 36 kDa fragments. For the native enzyme, they were represented only as minor components. These fragments could arise from protection of the cleavage sites in a cross-linked dimer structure or might represent a polypeptide of one kinase molecule cross-linked together with a fragment of another kinase molecule. To distinguish these two possibilities, the kinase was first cleaved with trypsin to a different extent (see Figure 8A) and subsequently cross-linked by EDC. Under the conditions known to produce a 64 kDa fragment, we observed formation of a cross-linked polypeptide of 85 kDa (Figure 8C). In contrast, there was no cross-linkage when MLCKase was first hydrolyzed in the presence of Ca^{2+}/CM so that the 61 kDa (active and CM-independent) residual fragment was formed (Figure 8D). This suggested that the cross-linkage was formed due to the interaction between the 64 kDa tryptic fragment (inactive, containing the autoinhibitory domain; Ikebe at al., 1987) of one MLCKase molecule with the 24 kDa fragment of another molecule.

No polypeptide of 36 kDa was formed during the cross-linkage under these conditions. We can therefore conclude that the formation of MLCKase dimers (oligomers) resulted from interaction between the autoinhibitory domain located in the C-terminal part of the 64 kDa fragment (Ikebe at al., 1987) and the 24 kDa MLCKase tryptic fragment containing titin-like II-3 domain (Olson et al., 1990). This indicates that the regulatory domain of the kinase plays a role in the kinase oligomerization.

DISCUSSION

The present report demonstrates for the first time that smooth muscle myosin light chain kinase should be considered as an oligomeric enzyme and is not monomeric as has been assumed in previous studies [see Edelman et al. (1987), Hartshorne (1987), and Stull et al. (1993)]. Some preliminary observations describing these novel properties have been already published. These principal aspects were the following: (i) the cooperative activation of the kinase by calmodulin (Sobieszek, 1991); (ii) Ca^{2+}/CM-dependent possibly oligomeric inhibition of the kinase activity (Sobieszek et al., 1993); and (iii) Ca^{2+}/CM-dependent intermolecular autophosphorylation of the kinase (Sobieszek, 1994a). Thus, it is apparent that the activity of MLCKase is not simply activated by CM but can be modulated also by the interaction between kinase monomers with or without CM.

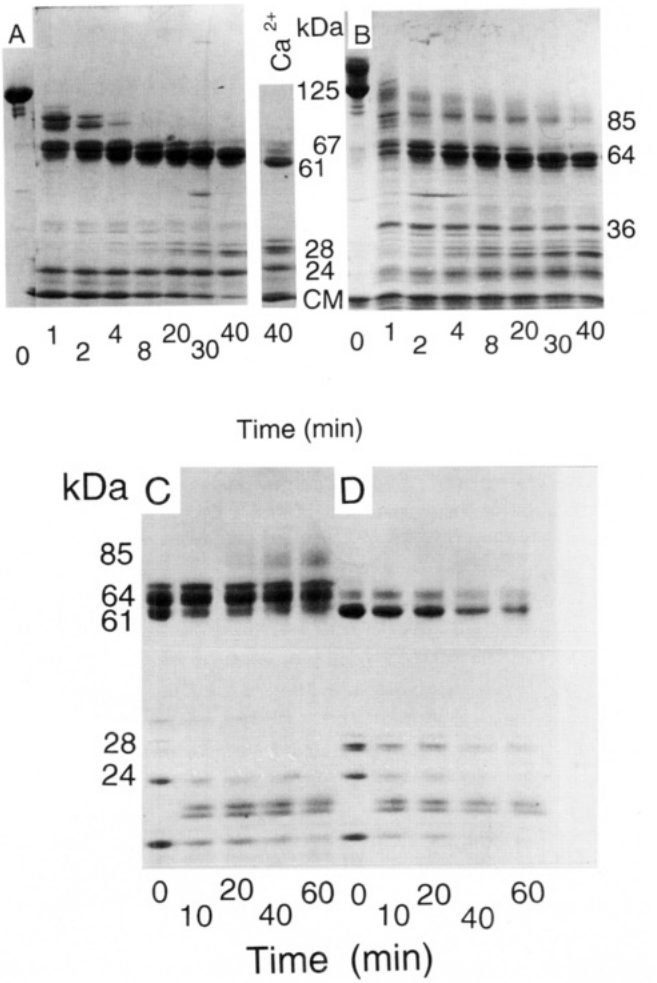


FIGURE 8: Identification of domain(s) responsible for formation of the kinase dimers and oligomers. (A) Control trypsinolysis of MLCKase. (B) The kinase cross-linked with EDC and subsequently hydrolyzed with trypsin. (C) The kinase hydrolyzed by trypsin (8 min; Ca^{2+} removed by EGTA) and subsequently cross-linked with EDC. (D) The kinase hydrolyzed by trypsin (40 min; Ca^{2+} present) and subsequently cross-linked with EDC. Ca^{2+} present during trypsinolysis was removed before the cross-linkage by EGTA. Note formation of the 85 kDa cross-linked polypeptide in (B) and its absence in (C).

The main evidence for the oligomerization of the kinase comes from the cross-linking experiments involving the use of the standard zero-length cross-linker EDC (Mornet et al., 1989). There were no significant modifications of the catalytic activity of the kinase by EDC, and those that were observed correlated with a Ca^{2+}/CM-dependent modification of the native enzyme. EDC treatment of the kinase resulted in formation of the cross-linked kinase dimers and oligomers.

The efficiency of the EDC cross-linkage was increased if sulfo-NHS was added to the medium. This indicates that the actual concentration of the kinase oligomeric forms was higher than that revealed by a simple EDC cross-linkage. This was confirmed by light-scattering measurements (Filenko et al., in preparation), demonstrating high levels of kinase dimers and oligomers at physiological or high ionic strength conditions. Independently, in a sedimentation equilibrium study Ausio and colleagues (Ausio et al., 1992) also concluded that approximately 15% of the kinase exists in the dimeric form at 0.2 M salt.

EDC appeared to act very specifically since all the cross-linkage was associated with a relatively small 3 kDa polypeptide (representing about 3% of the MLCKase amino acid sequence and containing the autoinhibitory domain). In addition, as would be expected with an oligomeric process, formation of the cross-linked kinase forms depended on the kinase concentration and ionic conditions of the medium. Other data supporting the oligomerization hypothesis come from the binding exhibited by free CM and CM cross-linked to the kinase during affinity chromatography on a CM-affinity column (Figure 7), as well as by the classical gel filtration experiments reported previously (Sobieszek, 1991).

Formation of the cross-linked kinase species implies the presence of a relatively strong interaction between the monomers to form dimers and between protomers within the oligomers. These interactions govern the equilibrium between the three kinase species which, as we have shown, was affected by the presence of Ca^{2+}/CM. The observed shift in the direction of dimers on addition of Ca^{2+} (when CM was present) points to possible pathway(s) for the modulation of the kinase activity. As indicated by our activity data, a reduction in the affinity of the kinase for calmodulin (Sobieszek et al., 1993) as well as a 2-fold reduction of the apparent rate of the kinase reaction was observed for the kinase dimers.

The oligomer-dependent modification of the kinase affinity for CM demonstrated here seems to be similar to that demonstrated for erythrocyte Ca^{2+}-ATPase, another Ca^{2+}/CM-dependent enzyme. As shown by Kosk-Kosicka et al. (1990), the latter formed oligomers in a concentration-dependent manner. In addition, the oligomers were shown to bind CM with much higher affinity than the monomers. The main difference between the kinase and the Ca^{2+}-ATPase lies in the regulation of the equilibrium between the oligomeric forms. For the Ca^{2+}-ATPase, the equilibrium does not appear to be affected by Ca^{2+}/CM whereas the kinase oligomer−dimer equilibrium was strongly affected by the presence of these ligands. Analogous changes were observed for muscle phosphofructokinase (PFK), an enzyme that plays a central role in the regulation of glycolysis but which has been identified as a CM-binding protein (Mayr & Helmeyer, 1983). In the subsequent extensive time dependence activity studies, taken together with the light-scattering data of Mayr (1984a,b) it was concluded that dissociation of active PFK tetramers to inactive dimers is induced by strong binding of CM to the dimers, resulting in a decrease in PFK catalytic activity. These properties of PFK are similar to those described for smooth muscle MLCKase. One obvious difference between the two enzymes, however, is that PFK binds CM with high affinity only in its dimeric form whereas in the case of MLCKase the oligomers appear to have a higher affinity for CM than the dimers. This relative high affinity of the oligomers was also observed in our CM to kinase binding experiments using gel filtration chromatography and radioactively labeled CM (Sobieszek, 1991).

Formation of the kinase oligomers at low, and their disappearance at moderate, salt concentrations suggests the presence of oppositely charged domains within the kinase molecules having high affinity to each other and possibly to other proteins. This is consistent with the difference between smooth and skeletal muscle MLCKases. It has been noted previously that the skeletal muscle enzyme is readily lost during myofibril preparation (Stull et al., 1985; Sobieszek, unpublished observation). In contrast, smooth muscle MLCKase binds tightly to thick myosin-containing filaments (Sobieszek, 1990). Perhaps this tight binding should be expected from the amino acid sequence of the smooth muscle enzyme. As pointed out by Olson et al. (1990), there are fascinating similarities in the amino acid sequence of the kinase, the *unc*-22 gene product of *Caenorhabditis elegans*, and titin. The latter two are known to be involved in the maintenance of myosin filaments (Kiff et al., 1988). Our data suggest that the C-terminal titin-like domain II-3 of the kinase may be involved in oligomer formation. The oligomerization of the kinase might in turn affect the MLCKase binding to myosin and, consequently, modulate phosphorylation of myosin.

Up to this time there is an accumulation of data indicating that the Ca^{2+}/CM-dependent activation of MLCKase is not the only Ca^{2+}-dependent regulatory mechanism of smooth muscle contraction. Dillon et al. (1981) demonstrated that in the case of muscles contracted with KCl myosin phosphorylation significantly decreased, following an initial increase, while the force increased constantly to a maximum. The second, frequently discussed phenomena is the so-called "Ca^{2+}-sensitivity of myosin phosphorylation" (Rembold, 1992; De Lanerolle & Paul, 1991). Several investigators have hypothesized that large increases in Ca^{2+} per se may decrease the sensitivity of the contractile apparatus to Ca^{2+} (Stull et al., 1990; Kitazawa & Somlyo, 1990). Different mechanisms, including Ca^{2+}-dependent regulation linked to thin filaments as well as Ca^{2+}-dependent phosphorylation of the MLCKase by other protein kinase(s), were proposed to explain this observation [for a review, see de Lanerolle and Paul (1991) and Rembold (1992)]. An alternative explanation would be that the disassembly of kinase oligomers and the formation of less active and less Ca^{2+}/CM-sensitive dimers (during Ca^{2+} transient, following the initiation of contraction) may decrease the level of myosin phosphorylation and at the same time reduce the kinase sensitivity to Ca^{2+}. Studies are in progress to substantiate this interesting hypothesis.

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