Ca²⁺–calmodulin-dependent modification of smooth-muscle myosin light-chain kinase leading to its co-operative activation by calmodulin

Apolinary SOBIESZEK, Anna STROBL, Birgit ORTNER and Eduard B. BABIYCHUK

Institute of Molecular Biology, Austrian Academy of Sciences, Billrothstrasse 11, A-5020 Salzburg, Austria

It has recently been shown that at relatively high molar ratios of myosin light-chain kinase (MLCKase) to calmodulin (CM) almost complete inhibition of the kinase activity occurs [Sobieszek (1991) *J. Mol. Biol.* **220**, 947–957]. This inhibition resulted in a highly co-operative activation of MLCKase by CM, whereas the opposite activation (of CM by kinase) was hyperbolic, as expected (unco-operative). This difference in activation was observed only for kinase preparations preincubated with sub-stoichiometric amounts of CM, and only when micromolar concentrations of Ca²⁺ were present. The inhibitory effect was variable and depended not only on the concentration ratio of kinase to CM but also on the MLCKase preparation. For most of the preparations full inhibition required 5–15 min of preincubation at 25 °C and a 3–6-fold molar excess of kinase

over CM. The inhibition was reversible, since full activity could be obtained after saturation of the kinase by additional CM. The inhibitory effect did not require ATP (excluding phosphorylation-type modifications of the kinase), and dephosphorylation of the kinase was not involved, since inhibition of an endogenous MLCK phosphatase by microcystin-LR did not decrease the inhibitory effect. Since the co-operative activation by CM was observed for cross-linked MLCKase preparations enriched in kinase dimers, but was absent for the analogous preparations enriched in the oligomers, we concluded that Ca²⁺–CM-dependent changes in the oligomeric state of the kinase were responsible for the modification observed. The exact nature of these modifications remains to be established.

INTRODUCTION

Myosin light-chain kinase (MLCKase) is a calmodulin (CM)-dependent enzyme responsible for the regulation of actin–myosin interaction in smooth muscle. In the presence of Ca²⁺ the enzyme phosphorylates the regulatory light chain of myosin, permitting a cyclic interaction of myosin heads with actin filaments (for reviews see Kamm and Stull, 1985; Marston, 1982; Adelstein and Eisenberg, 1980; Small and Sobieszek, 1980). The kinase, being completely inactive in the absence of CM and Ca²⁺, represents the primary molecular switch leading to contraction of this muscle type. In view of its significance, there have been numerous studies aimed at determining the molecular mechanism by which CM activates this target enzyme. Two types of models have been suggested. In the first, a major conformational change of the kinase takes place during its association with the Ca²⁺–CM complex (see review by Kennelly et al., 1989). However, attempts to detect this conformational change by fluorescence (Malencik et al., 1982), proteolysis (Walsh, 1985) or physical methods (Adelstein and Klee, 1981) have indicated that only a small change of overall enzyme conformation occurs upon activation. Such a conformational change would be of significance if it were restricted to the kinase active site, but this has not been shown. In the second model of activation, the kinase activity is inhibited by a pseudosubstrate autoinhibitory domain, homologous to the phosphorylation site on the myosin regulatory light chain, and binding of CM activates the enzyme by removing the inhibition. The main evidence in support of this model comes from the observation that synthetic peptides modelled after the CM-binding region of the kinase act as potent inhibitors of its activity (Kemp et al., 1987; Kennelly et al., 1987).

Although the autoinhibitory model appears to be widely accepted, not only for the MLCKase but also for several other CM-dependent enzymes such as plasma-membrane Ca²⁺ pump

(Vorherr et al., 1990), Ca²⁺–CM-dependent protein kinase II (Colbran et al., 1988) or calcineurin (Hashimoto et al., 1990), the collected data are largely inferential. More recent observations on the binding of mutant CMs by the kinase without its activation (VanBerkum and Means, 1991) indicate that, besides the autoinhibition, an additional conformational change may be necessary. In addition, our own recent data indicate that MLCKase may exhibit allosteric properties (Sobieszek, 1991). Specifically, its activation by CM proves to be positively co-operative and not hyperbolic, which would be expected from the 1:1 binding stoichiometry. This co-operativity, having important physiological implications, could not be explained readily in kinetic terms. In the present report we present data which support the concept that the unexpected co-operativity can be accounted for by concentration-dependent (oligomeric) effects, leading to lowering of the kinase's affinity for CM.

MATERIALS AND METHODS

MLCKase and its activator CM were purified from turkey gizzard or pig stomach muscle (as previously described by Sobieszek and Barylko, 1984; see also Sobieszek, 1991). In the latter stages of the present study a modification of the MLCKase purification procedure was introduced. The 40–55% satd.-(NH₄)₂SO₄ fraction of the kinase (usually temporarily frozen in liquid nitrogen and stored at –70 °C) was directly applied to a DEAE-Sepharose 6B-CL column. The omitted gel-filtration step was often carried out as a final step after CM-affinity chromatography. For this purpose a small-size (1.0 cm × 60 cm) Sephacryl S-200 (Pharmacia, Uppsala, Sweden) column was used. In view of the solubility properties of the kinase (see the Results section), the column was equilibrated with our AA buffer (see below) containing 0.3 M NaCl. This purification step, however, did not give significant improvement in the purity of the

kinase preparations, confirming their high degree of homogeneity. MLCKase concentration was measured from its absorption at 278 nm by using the absorption coefficient $A^{1\%} = 1.1$ (Adelstein and Klee, 1981).

Myosin or its regulatory 20 kDa light chain was used as a MLCKase substrate. Myosin was purified from freshly extracted actomyosin (Sobieszek and Bremel, 1975) by $(\text{NH}_4)_2\text{SO}_4$ fractionation (Sobieszek, 1985) and stored at -70°C after initial freezing in liquid nitrogen. This preparation contained relatively high amounts of endogenous MLCKase and CM; if necessary they were removed by chromatography on Phenyl- and CM-affinity columns arranged in tandem (Sobieszek, 1990). The myosin regulatory light chain was isolated by the method of Perrie and Perry (1970) and purified as described previously (Sobieszek, 1988). Concentrations of myosin and its LC₂₀ light chain were measured by the biuret method (Gornall et al., 1949).

For 'freezing' and separation of different oligomeric species, the kinase was first cross-linked by addition of 2–5 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDC) and incubation for 20 min at 25°C . The reaction was terminated by addition of a 2-fold molar excess of dithiothreitol, and the kinase was subjected to gel filtration on a Sephacryl S-200 column ($0.6\text{ cm} \times 100\text{ cm}$), which was equilibrated with the assay buffer containing in addition 0.3 M NaCl. Dimerization and oligomerization of the kinase was analysed by mini-SDS-gel PAGE essentially as described by Matsudaira and Burgess (1978), using 3% stacking gel and a gradient (9–20%) separating gel.

The phosphorylation rate measurements were carried out at 25°C in AA buffer of the following composition (in mM): KCl 60, MgCl_2 2, dithioerythritol 0.5 and imidazole 10, adjusted to pH 7.5 at 4°C . All other details were given previously (see Sobieszek, 1991) or in the corresponding Figure legends.

RESULTS

It has been shown recently that smooth-muscle MLCKase is activated by CM with a high degree of positive co-operativity (Sobieszek, 1991). This co-operativity was not observed for activation of CM by the kinase; on the contrary, at constant CM concentration the dependence of the kinase activity on its concentration was biphasic. As shown in Figures 1 and 2, at the lowest range of kinase concentrations, the kinase activity increased steeply until the CM present became saturated by kinase. The activity then decreased so that, at about 4–6-fold excess of the kinase apoenzyme relative to CM, the activity was inhibited to very low levels. One would expect that after saturation the activity should remain constant irrespective of the apoenzyme concentration. Since the activity of MLCKase has an absolute requirement for CM, the observed inhibition appears to point to another, and perhaps more clear, demonstration of an unidentified allosteric-type inhibition, leading to its positively co-operative activation by CM (see below).

Significantly, the inhibition was also observed with intact myosin as substrate and with myosin containing (Figure 1a) as well as free from (Figure 1b) endogenous MLCKase and CM. For the former case, however, partial removal of the kinase on a CM affinity column was necessary, since the endogenous phosphorylation rates would have been too high. For the experiments carried out with myosin, the degree of inhibition obtained was in the range 50–75%. The inhibitory effect described above tended to be rather variable (compare Figures 2a and 2b). On the one hand, the apoenzyme/CM ratio at which maximal inhibition occurred varied significantly; on the other hand, the extent of inhibition was strongly affected by the range of the MLCKase concentrations used. The latter range, in turn,

depended on the concentration of CM present. Consequently, the clearly biphasic curves accompanied by severe inhibition required more extensive experimentation. The main difficulty was that relatively high MLCKase concentrations were necessary for demonstration of the inhibitory effect. Inevitably the phosphorylation rates were high, and sometimes it was necessary to use inconveniently short assay times (15–30 s). In addition, the presence of even low levels of a Ca^{2+} -CM-independent activity would, at this high kinase concentration, mask the inhibition. Considering all these factors, it was not surprising that, even with the regulatory light chain as the substrate, kinase preparations

Figure 1 Biphasic relationship between MLCKase activity and its concentration at constant CM levels

Note that after saturation of CM the rate decreased to relatively low levels and did not remain constant, as would be expected from classical kinetic considerations. This inhibition at high kinase/CM molar ratio is called the 'inhibitory effect'. The preparations used were from turkey gizzard muscle. In (b) myosin, used as a substrate, was free of endogenous MLCKase and CM (Sobieszek, 1990), whereas in (a) it contained endogenous CM, but some of its endogenous MLCKase was removed to decrease initial phosphorylation rates. The rates are given in c.p.m./min, and myosin concentrations were 23 and $24 \mu\text{M}$ in (a) and (b) respectively.

Figure 2 Ca^{2+} /CM-dependence of the inhibitory effect

As in Figure 1, MLCKase activity was measured as a function of its concentration and at constant CM, but isolated regulatory light chain (LC_{20}) was used as a substrate. Note that the activity was inhibited up to very low levels when the kinase concentration exceeded 3–4-fold that of CM ($\diamond$). Under exactly the same conditions, the inhibition was not observed when the assays were initiated by addition of MLCKase ($\times$). Thus the inhibitory effect resulted from a preincubation of the kinase with CM before the phosphorylation assays (see also Figure 4). Preincubation of kinase and CM with Ca^{2+} removed by EGTA (0.2 mM) produced very little inhibition ($\blacklozenge$). In the latter case Ca^{2+} was added with ATP to initiate the assays. The effect was not modified by addition of $0.5 \mu\text{M}$ microcystin-LR during the preincubation and phosphorylation assay ($\bullet$), indicating that no dephosphorylation of the MLCKase was involved. In (a) pig stomach preparations were used and CM concentration was $0.3 \mu\text{M}$. In (b) the kinase and CM were from turkey gizzard, and CM concentration was $0.15 \mu\text{M}$.

were not fully inhibited (Figure 2b). The significance of this 'residual' activity remains to be investigated.

In further experiments we observed that the inhibitory effects depend on the order of addition of the assay components. The phosphorylation assay was normally initiated by addition of radioactive ATP, but when CM was added to the ATP no

Figure 3 Reversibility of the inhibitory effect

The progress of LC_{20} phosphorylation was monitored for two identical assay mixtures for which phosphorylation was initiated by addition of ATP to kinase preincubated with CM ($\times$) or by addition of LC_{20} with CM ($\diamond$). Note that, although rates were initially very different, they became similar after addition (at 1 min) of saturating concentrations of CM. Assay conditions were as in Figure 2(a), but the kinase/CM ratio was 15:1.

inhibition was observed. Similarly, no inhibition was observed after initiation of the assays with a solution containing the substrate (isolated regulatory light chain of myosin) together with CM. After testing all possible sequences of reagent addition, it was concluded that the inhibition resulted from a preincubation of the MLCKase with sub-stoichiometric concentrations of CM before initiation of the phosphorylation assays. This is demonstrated in Figure 2, where, with CM added to ATP, no inhibition was observed, and as expected from classical kinetic considerations the kinase activity remained constant at its high saturation levels (Figure 2; $\times$). Thus it is implicit that some CM-dependent changes of the MLCKase were taking place during the 30–60 min pipetting time (carried out on ice) resulting in the inhibition. Figure 2(a) shows also that these changes require the additional presence of micromolar concentrations of Ca^{2+} . Preincubation of the kinase with CM, but with Ca removed by EGTA, produced no inhibition. In that case, additional Ca^{2+} was added to saturate the EGTA during the phosphorylation-rate measurements. The 25% inhibition observed in Figure 2(a) most likely resulted from a slightly higher proteolytic degradation of the pig stomach kinase in comparison with that of gizzard enzyme.

The inhibition of MLCKase activity was fully reversible. The corresponding data are shown in Figure 3, where it was demonstrated that the presence of high (stoichiometric) CM concentrations in the differently initiated assay tubes resulted in approximately the same final phosphorylation rates and the same maximal phosphorylation levels. Thus modified and unmodified forms of the kinase have similar properties at stoichiometric ratios of CM and kinase.

Having established that the inhibitory effect resulted from a Ca^{2+} -CM-dependent and ATP-independent modification of the kinase, it was necessary to show the time range required for these

Figure 4 Dependence of MLCKase inhibition on the time of preincubation with Ca^{2+} /CM, and absence of this inhibition for the same kinase preincubated at high concentration

MLCKase phosphorylation rates were measured by addition of kinase preincubated with CM for the times plotted at the abscissa ($\diamond$). In (a) pig stomach preparations were used, and the kinase (at $4.0 \mu\text{M}$ concentration) was preincubated with $0.12 \mu\text{M}$ CM. In (b) the kinase and CM were from turkey gizzard, and their concentrations were 3.5 and $0.65 \mu\text{M}$ respectively. The inhibitory effect was not observed after preincubation of kinase and CM (at the same molar ratio) at 12-fold higher concentrations ($\blacklozenge$) with the same final concentrations. Thus it appears that oligomeric-type modification(s) were involved. In contrast, addition of $2.5 \mu\text{M}$ okadaic acid did not affect kinase modification ($\times$), excluding dephosphorylation-type effects (see the text).

changes to occur. This could readily be measured by varying the preincubation time of the kinase with CM while the subsequent rate measurements were carried out, in the usual manner, at a constant time. Naturally the MLCKase and CM concentrations and their molar ratio were maintained constant and corresponded to those for very high inhibition. As shown in Figure 4(a), pig stomach MLCKase required about 4 min preincubation with

Figure 5 Co-operative activation of MLCKase after its preincubation with calmodulin

In (a) pig stomach kinase was used at a concentration of approx. $0.3 \mu\text{M}$, and in (b) turkey gizzard kinase concentration was $0.5 \mu\text{M}$. Without modification the activation was essentially hyperbolic ($\circ$), whereas after modification the activation curves became sigmoid. Hill coefficients (h) were calculated by an empirical formula using total and not free CM (Sobieszek, 1991).

CM at 25°C for it to be modified. In general, similar turkey gizzard preparations required rather longer preincubation times (Figure 4b), but this time rarely exceeded 10–15 min.

The physiological consequence of the observed inhibitory effects became apparent on examination of the standard CM-activation curves which are normally obtained at constant MLCKase and variable CM concentrations. As shown in Figure 5, without preincubation the kinase is activated by CM in an approximately hyperbolic manner, the activation curve exhibiting a Hill coefficient (h) of about 1. Preincubation of the kinase with CM resulted in co-operative activation. The observed increase in co-operativity between the unmodified and modified forms was about 2.0-fold, but the increase could be even greater since, on the one hand, the preparations tested exhibited a degree of co-operativity before preincubation (Figure 5b) and, on the other, most of the CM-activation curves obtained in the normal assays exhibited h values in the range 2.5–3.5 (see Sobieszek, 1991).

The observed change(s) in MLCKase properties took place in the absence of ATP; therefore a phosphorylation-type covalent modification can be excluded. In contrast, dephosphorylation of the kinase by a CM-dependent protein phosphatase was one of the first possibilities that was considered. This was of particular relevance, since an apparently CM-dependent protein phosphatase contaminating the MLCKase preparations has been identified (A. Sobieszek, unpublished work). However, addition of $0.7 \mu\text{M}$ microcystin-LR or of $2.7 \mu\text{M}$ okadaic acid, which are phosphatase inhibitors, during the preincubation did not affect the inhibitory action (Figures 2b and 4b respectively), although the inhibitors were highly effective in inhibiting the contaminating

Figure 6 Inhibitory effect and MLCKase phosphorylation

Phosphorylation rates of unphosphorylated ($\diamond$) and autophosphorylated ($\blacklozenge$) turkey gizzard kinase were measured as described in Figure 2. Note that both rates were almost identical. CM added during autophosphorylation was removed by a gel-filtration step on a S-200 column. The unphosphorylated kinase was also subjected to the same purification step.

protein phosphatase. Thus it can be concluded that dephosphorylation by this endogenous protein phosphatase was not involved.

In an independent line of investigations (A. Sobieszek, unpublished work), we have established that MLCKase undergoes a relatively slow Ca^{2+} -CM-dependent autophosphorylation. It was therefore considered important to examine whether or not this self-phosphorylation was involved in the observed modification. For this purpose Ca^{2+} , CM and microcystin-LR were added to the kinase, which phosphorylated for 45 min in the presence of 0.5 mM ATP. After removal of the additions by gel filtration on a Sephacryl S-200 column (see the Materials and methods section), the kinase was stably autophosphorylated and could be used for determination of its self-inhibitory properties. As shown in Figure 6, inhibition of the kinase at high concentration was practically identical with that of unphosphorylated kinase, the latter kinase being also subjected to the same gel-filtration step. This type of experiment indicates not only that kinase autophosphorylation was apparently not involved, but also points again to a lack of any effects of the endogenous phosphatase in the observed inhibition.

As indicated above, the modification of MLCKase depended strongly on its concentration. The kinase when preincubated with CM at 10–15-fold higher concentration, but at the same ratio (to CM), and subsequently diluted during the assay did not undergo the same modification as diluted kinase (Figure 4b; $\bullet$). This indicates that the modification(s) involved could be of an allosteric type, or at least that they were affected by the oligomeric state of the kinase.

Oligomerization of MLCKase under the ionic conditions of the assay was apparent from its absorption spectrum (A. Sobieszek, unpublished work). At high concentrations (200–

Figure 7 Dimerization of MLCKase and partial separation of the dimeric and oligomeric species

(a) Turkey gizzard kinase (at 12 μM concentration) was first cross-linked with EDC and then subjected to gel filtration on a Sephacryl S-200 column as described in the Materials and methods section. The 'ascending' and 'descending' fractions (hatched) were then analysed for their activation by CM (Figure 8). (b) Separation of the MLCKase species by the S-200 column (Figure 7a) as revealed by SDS/PAGE. Lanes: a, unmodified kinase; b, cross-linked kinase before its application on the S-200 column; c, d, e, f respectively, tubes 21, 23, 25 and 27 of the S-200 column; g, M_r standards. Note the presence of a strong MLCKase dimer band at approx. M_r 190 000 and a diffuse high- M_r band corresponding to the kinase oligomer (olig.); mon, monomer.

400 μM), the kinase appeared soluble (as stored) at 60 mM KCl/25% glycerol in AA buffer, but it formed a fine suspension after 10–20-fold dilution in the same buffer containing no glycerol. Addition of 120 mM NaCl (or KCl) resulted in solubilization of the kinase and clarification of the suspension.

The direct involvement of oligomeric-type modifications was demonstrated by cross-linking experiments involving MLCKase and EDC. The EDC is a zero-length cross-linker commonly used in studies on actin–myosin interaction (see Mornet et al., 1989). Using 2–5 mM EDC we were able partially to 'freeze' the oligomer–monomer or oligomer–dimer equilibrium of the MLCKase which exists at high concentrations in the region above 5 μM . Under normal assay conditions (60 mM KCl and pH 7.0) and for 30 min incubation, EDC produced relatively low losses of kinase activity, amounting to a 5–15% decrease in the absence and a 15–25% decrease in the presence of Ca^{2+} and CM.

Figure 8 Co-operative and hyperbolic activation of dimeric and oligomeric species of MLCKase

Turkey gizzard kinase was first cross-linked by EDC (a zero-length cross-linker) and then chromatographed on the S-200 gel-filtration column (Figure 7a). The 'ascending' (●) and 'descending' (○) S-200 column fractions (see Figure 7b) were examined for their activation by CM. Note the significant change in the Hill coefficient for the kinase preparation enriched in dimeric ($h = 1.9$) in comparison with that enriched in the oligomeric ($h = 1.0$) species.

In order to obtain partial separation of the different oligomeric forms, the cross-linked kinase was fractionated on a Sephacryl S-200 gel-filtration column as described in the Materials and methods section (Figure 7a). The result was a slight broadening, on the high- M_r side, of the cross-linked kinase peak, compared with normal kinase. Significantly, SDS/PAGE (Figure 7b) revealed that the kinase from 'ascending' fractions was enriched in the oligomer, whereas the 'descending' fractions contained mainly the dimers and monomers. Complete separation of the species was not expected, but the CM activation properties of the kinase eluted in 'ascending' and 'descending' fractions were clearly different (Figure 8). Unexpectedly, the 'ascending'-fraction kinase, which was previously concluded to be eluted in the form of large oligomers (Sobieszek, 1991), was activated in a hyperbolic manner. In contrast, the 'descending'-fraction kinase showed positive co-operativity, with h approx. 2. Thus co-operativity of MLCKase relative to CM resulted from an apparent decrease in the affinity of kinase dimer for CM. Binding of CM to the kinase oligomers appeared to shift the equilibrium in the dimer-monomer direction, since for the kinase cross-linked in presence of CM the diffuse oligomer band became weaker or absent. Taken together, these data demonstrate that oligomeric-type modifications were involved in the observed changes of the MLCKase activation by CM.

DISCUSSION

In the present studies we have extended our previous investigations on the inhibition of MLCKase activity at sub-stoichiometric ratios to CM (Sobieszek, 1991). Originally, inhibition was observed for systems comprising purified MLCKase and isolated regulatory light chain as substrate. A similar inhibition has now been demonstrated for the native substrate system (intact filamentous myosin) with or without its endogenous activator (CM). Thus it is considered highly likely that an analogous inhibitory state of the kinase exists for the myosin system *in vivo*. A similar conclusion can be made when one

considers the functional consequence of the inhibition, namely a significant increase in the co-operative activation of MLCKase by CM. Taken together, we suggest that, in addition to free Ca^{2+} , cytoplasmic CM levels may be involved in the regulation of MLCKase activity *in vivo*.

An inhibition leading to a co-operative activation implies the presence of an allosteric inhibitor. However, such an inhibitor has not been identified in the present study: on the contrary, we have shown that the inhibition results from a modification of the kinase in the absence of ATP, normally taking place before the phosphorylation assays. The modification was found to require not only sub-stoichiometric concentrations of CM, but also the presence of micromolar levels of Ca^{2+} , which would be expected for a CM-dependent process. We have not established the exact mechanism of the modification(s) involved, and this no doubt interesting but difficult problem remains to be investigated. However, some types of changes can be excluded, such as modifications involving phosphorylation which would require the presence of ATP. In this context it was observed that addition of ATP to the kinase and CM was not necessary for the effect to take place. Similarly, preincubation with Ca^{2+} and CM in the presence of added ATP resulted in MLCKase auto-phosphorylation, but did not modify the inhibitory effect. The phosphorylated and unphosphorylated kinase exhibited the same inhibitory properties.

The dephosphorylation-dependent modifications, namely Ca^{2+} -CM-dependent dephosphorylation of the kinase, was considered a more likely possibility, since in parallel studies it was demonstrated that MLCKase preparations can be contaminated by an CM-dependent endogenous protein phosphatase (A. Sobieszek, unpublished work). This phosphatase, which occurs in extremely low amount (1:1000 molar ratio to kinase), could be inhibited by okadaic acid and microcystin-LR, but addition of these inhibitors did not affect the inhibitory properties of the MLCKase apoenzyme. Correspondingly, the dephosphorylation of the kinase by this protein phosphatase did not appear to be involved. However, in view of the presence of multiple phosphorylation sites on the kinase (see Stull et al., 1990), with their dephosphorylation being affected by CM, a possible dephosphorylation of the kinase leading to its inhibition cannot be rigorously excluded. Inhibition resulting simply from the presence of the endogenous phosphatase, however, can be excluded, since such an inhibition would not depend on the observed 5–10 min preincubation.

Independent of the nature of the MLCKase modification involved, it is apparent that after modification most of the previously active kinase adopted an inhibitory form. Perhaps this state bears some similarity to the analogous state of the multifunctional Ca^{2+} -CM-dependent kinase that is present in brain. This kinase forms oligomers with 8–12 similar subunits which are autophosphorylated intermolecularly, resulting in conversion of the enzyme into a partially Ca^{2+} -CM-independent form (see Hanson and Schulman, 1992). We consider that this latter state may be analogous to the inhibitory state of the MLCKase described here, but its function would be to increase the co-operativity of the CM activation. The regulatory mechanism of the multifunctional kinase II includes also a Ca^{2+} -CM-dependent autophosphorylation which results in trapping of CM by the kinase (Meyer et al., 1992). Similar CM trapping does not take place with the MLCKase, but a 6-fold increase in the CM 'off' rate constant at the high kinase/CM ratios has been observed (T. Meyer and A. Sobieszek, unpublished work). Since no pronounced effects of autophosphorylation on the kinase properties could be demonstrated (A. Sobieszek, unpublished work), it is clear that more studies will be necessary to understand the

possible relationships between the observed covalent and non-covalent modifications of the MLCKase.

From the initial cross-linking experiments presented here, it is clear that MLCKase can form dimers and oligomers and that in solution these two forms are in equilibrium with the kinase monomers. The apparent affinities of the three forms for each other were in the micromolar range, in contrast with the nanomolar affinities observed for the kinase-CM interaction. From the observed increase in co-operativity, it is apparent that the latter affinity was decreased for the dimeric form of the kinase in comparison with that of the monomers or oligomers. More important, however, are the parallel changes in the 'off' and 'on' rate constants. Although determination of these constants requires a separate study, we consider it likely that the decreased affinity results from a slowing of the 'on' rate of kinase for CM or from an increase in the 'off' rate constant. A 6-fold increase in the latter constant observed (T. Meyer and A. Sobieszek, unpublished work) is consistent with our data. The increase might be even higher *in vivo*, where both the kinase and CM appear to be tightly bound to filamentous myosin (Sobieszek, 1985, 1990). Accordingly, CM activation of the kinase system with filamentous myosin as a substrate was seen to exhibit a higher degree of co-operativity than was observed for the isolated light-chain system (Sobieszek, 1991).

The oligomer-dependent increase in the co-operativity of CM activation proposed here could represent a similar regulatory pathway to that demonstrated for another Ca^{2+} -CM-dependent enzyme, namely erythrocyte Ca^{2+} -ATPase (see Kosk-Kosicka et al., 1990). This enzyme exhibits a concentration-dependent activation, in addition to the normal activation by Ca^{2+} -CM. In the former case, three independent methods, including Ca^{2+} -ATPase activity, polarization fluorescence and fluorescence resonance energy transfer, have established that concentration-dependent activation is due to oligomerization of the enzyme. The oligomers bind CM with lower stoichiometry and higher affinity and exhibit higher co-operativity than the monomeric form. With respect to CM binding, this agrees with our previous CM-binding data (Sobieszek, 1991). At the same time our previous interpretation that the larger oligomeric forms of the kinase are not active does not agree with the present data. Instead, it appears that all the forms were active, but they differed in their affinity for CM. Such an interpretation would require similar CM-binding properties for the native and the cross-linked oligomers. The response of these two types of oligomer to CM binding cannot be the same, since the cross-linked forms do not dissociate. A CM-dependent shift of equilibrium towards dimers and monomers was apparently taking place, since when CM and Ca^{2+} were present during the cross-linking the diffuse oligomer band became weaker or absent. Thus CM present at sub-stoichiometric concentrations relative to

kinase could also regulate the equilibrium between the three oligomeric forms of the kinase. Formation of the oligomers or even dimers could take place by a general mechanism of oligomerization for Ca^{2+} -CM-dependent enzymes, as suggested by Jarrett and Madhavan (1991), which involves the CM-like and the authentic CM-binding sites that appear to be present on all Ca^{2+} -CM-dependent enzymes. These sites are certainly present on MLCKase.

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