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Regulation of Smooth Muscle Myosin Light Chain Kinase

Allosteric Effects and Co-operative Activation by Calmodulin

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(Received 1 February 1991; accepted 13 May 1991)

The activation of smooth muscle myosin light chain kinase (MLCKase) by calcium and calmodulin (CM) was investigated over a wide range of concentrations of the enzyme using myosin (MY) or its isolated phosphorylatable light chain (L₂₀) as substrates. The enzyme showed allosteric behavior. The specific phosphorylation activity was dependent on the concentration of MLCKase as well as on the concentrations of both substrates. However, at the lower (nanomolar) range of kinase the corresponding substrate rate relationships were hyperbolic. A high positive level of co-operativity of kinase was also observed for activation by CM in the presence of Ca²⁺. There was a pronounced CM/Ca-dependent inhibition of MLCKase activity when its molar ratio to CM was four to one or more. These kinetic data suggested that MLCKase could exist in several oligomeric forms, with an inactive high molecular size form and an active low molecular size form (protomers and/or dimers). This conclusion was confirmed by gel filtration studies. CM was not directly involved in the oligomerization process but instead, the oligomeric kinase shared an increased affinity for CM.

Keywords: smooth muscle myosin light chain kinase; calmodulin; allosteric effects; co-operative activation.

1. Introduction

Myosin light chain kinase (MLCKase†) plays a central role in Ca-dependent regulation of vertebrate smooth muscle (for reviews see Kamm & Stull, 1985; Marston, 1982; Small & Sobieszek, 1980). In the presence of Ca²⁺ this enzyme phosphorylates a regulatory light chain of myosin enabling cyclic interaction of myosin with actin and subsequent tension development. Despite the general acceptance of the need of myosin phosphorylation for the activation of contraction, the extent of light chain phosphorylation has not been correlated with the magnitude of force generation (Kamm & Stull, 1985). In consequence the precise role of myosin phosphorylation, and hence that of MLCKase, in intact muscle needs to be clarified.

Smooth muscle MLCKase is a calmodulin-dependent enzyme that, in contrast to other enzymes of this type, is fully inactive in the absence of Ca²⁺ (Adelstein & Klee, 1981; Sobieszek & Barylko, 1984). In the presence of Ca²⁺, calmodulin (CM) forms a one-to-one complex with kinase, resulting in its activation. A mechanism has been suggested for this activation (Means & George, 1988) based on an analogy between the amino-terminal end of the CM binding portion of the apoenzyme and the substrate. In their model, the binding of CM near the pseudosubstrate results in structural changes to kinase. This, in turn, induces release of the pseudo-substrate and exposes the active-site for interaction with external substrates.

It was noted earlier (Sobieszek, 1985b) that in the presence of a fixed concentration of CM–MLCKase activity shows a biphasic concentration dependence. First the activity rises as the concentration of active CM–MLCKase complexes increases, reaches a maximum at a one-to-one CM to MLCKase ratio, and then subsequently declines as the concentration of kinase increases. This indicated that allosteric inter-

† Abbreviations used: MLCKase, myosin light chain kinase; MLCPase, myosin light chain phosphatase; CM, calmodulin; L₂₀, light chain of smooth muscle myosin, with relative molecular mass, 20 × 10³; MY, myosin; EGTA, ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid.

actions were involved in the regulation of the activity of this enzyme. The results presented here indicate that allosteric effects are indeed involved in the regulation of smooth muscle MLCKase.

2. Materials and Methods

(a) Protein and enzyme preparations

Unless otherwise stated all preparations were from turkey gizzard muscle. Myosin and CM-free myosin as well as MLCKase were purified as described previously (Sobieszek & Barylko, 1984; Sobieszek, 1985a, 1990). These preparations were stored in "AA" buffer (60 mM-KCl, 2 mM-MgCl₂, 0.5 mM-DTT and 10 mM-imidazole (pH 7.4 adjusted at 4°C) at -70°C after initial freezing in liquid nitrogen. Purification of the phosphorylatable myosin light chains was carried out as already described (Sobieszek, 1988) and the light chain was stored as a stock solution (20 mg/ml in AA buffer) at -30°C.

CM was purified from the 1st wash of muscle mince employed for the preparation of myofibrils. Tropomyosin and other major impurities were removed by addition of solid ammonium sulphate up to 62.5% saturation, followed by centrifugation. CM was then precipitated with 85% saturated ammonium sulphate and after pelleting, it was dissolved in a low ionic strength buffer containing 0.5 mM-EGTA. After dialysis and removal of the insoluble proteins, 0.5 mM-CaCl₂ and 15% ammonium sulphate were added and the clear supernatant applied onto a Phenyl Sepharose affinity column (Pharmacia, Uppsala, Sweden) at 4°C. The column was then extensively washed with the above buffer and subsequently eluted with the same buffer but in which Ca was replaced by 2 mM-EGTA. CM-containing tubes were identified using urea/glycerol polyacrylamide gel electrophoresis (Sobieszek & Jertschin, 1986) and their contents pooled into dialysis bags for a concentration/precipitation step with solid ammonium sulphate as described previously for the purification of the light chain of smooth muscle myosin, L₂₀ (Sobieszek, 1988). The dissolved CM-containing pellet was subjected to gel filtration on an AcA 54 column (LKB, France). A homogeneous preparation of CM was obtained after a subsequent ionic exchange/purification step, provided that the initial preparation contained only traces of tropomyosin. After another concentration/precipitation step with solid ammonium sulphate (as above) the dissolved pellet was dialyzed against AA buffer and stored at -30°C until use.

(b) Phosphorylation rate measurements

Phosphorylation assays were carried out as described previously (Sobieszek, 1989, 1990) except that the assay volume was increased to 125 µl in order to increase the accuracy of the rate measurements. Unless otherwise required the assays were carried out at saturating concentrations of [γ -³²P]ATP (0.75 to 1.5 mM) and L₂₀ (250 to 500 µM) and the assay times were chosen to insure a linear range of the ³²P incorporations. For the short incorporations (60 s or less) the reactions were terminated (while being mixed on a vortex) by the addition of 50 µl of saturated guanidine hydrochloride solution. In all 150 µl of this reaction mixture was spotted onto 2 cm × 4 cm pieces of Whatman 3MM chromatographic paper and these were dropped into 5% (w/v) TCA (trichloroacetic acid) solution containing 1 mM-potassium phosphate. For the longer incorporations, at the end of the assay time,

110 µl of the assay mix were spotted directly on the 3MM paper pieces and the reaction terminated by dropping the pieces into the TCA solution. The paper pieces from both types of assays were then processed identically for the radioactivity counts (Sobieszek, 1985a, 1990).

(c) Gel filtration and CM binding

In gel filtration experiments a Sepharose 6B-CL (Pharmacia, Uppsala, Sweden) column was used. The column (100 cm long and 0.6 cm in diameter) had been equipped with 2 flow adaptors and was equilibrated with AA buffer containing in addition 0.1 mM-CaCl₂. The sample volume was always approx. 500 µl and this corresponded to the volume of 1 fraction, or to 5 min of the application time (e.g. the flow rate was constant at 6.0 ml per h). The u.v. recorder was set at the highest sensitivity range that could provide low noise recordings and this was not changed for all the filtration and binding experiments. For the calibration runs, concentrations of marker proteins were adjusted so that the recorder gave approximately the same size peak as that obtained from very numerous kinase runs. The following relative molecular mass markers were used; thyroglobulin (M_r , 669 × 10³) from Sigma, St. Louis, U.S.A. and ferritin (M_r , 450 × 10³), catalase (M_r , 240 × 10³) and aldolase (M_r , 158 × 10³) all from Boehringer-Mannheim, Germany.

[¹²⁵I]CM-binding experiments were done using the same Sepharose 6B-CL column. The sample volume and MLCKase preparation were the same for a given binding experiment (a set of runs). Before each run the column was equilibrated with 40 to 50 ml of buffer (AA plus 0.1 mM-CaCl₂) containing a required concentration of [¹²⁵I]CM. After application of the sample, the same [¹²⁵I]CM buffer was used in the following run. Other conditions were as described above while CM-iodination was carried out as previously described (Sobieszek, 1990).

(d) Analysis of the kinetic data

The experimental data were analyzed using curve-fitting software made in collaboration with M. Smialek. The software† comprised a program for "minimalization" of a function of many parameters using a modified algorithm of Fletcher & Powell (1963) together with a function-plotting capacity. The model functions corresponded to the empirical Hill's equation (Segel, 1975) in estimations of the co-operativity coefficients, or to rate relationships with CM and Ca²⁺ concentrations being independent variables. After the introduction of initial values of the parameters a χ^2 function between experimental and theoretical values of the model function was then minimalized, now with the parameters as variables.

3. Results

(a) Allosteric regulation of MLCKase

(i) Specific activity and substrate effects

One of the characteristics of most allosteric enzymes is that they can exist in two or more oligomeric forms. This property is revealed in the

† A diskette containing the software together with the rate formula and other model functions used can be obtained from M. Smialek at a modest charge.

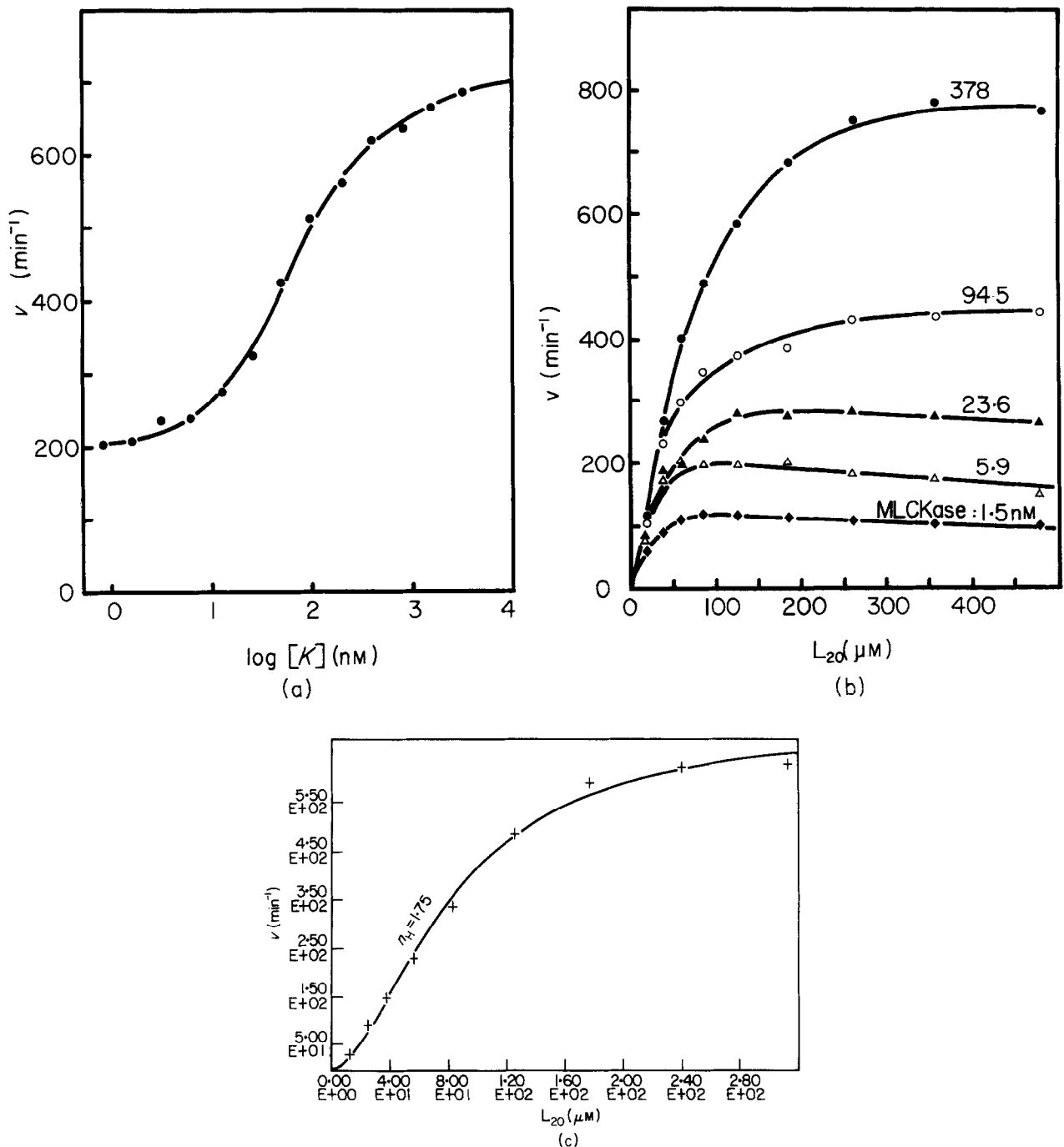


Figure 1. (a) Dependence of specific activity of MLCKase on its concentration. In the assays the CM to MLCKase ratio was kept constant at about 1.2 to 1. The assay time was 5 s for the highest kinase concentration and was increased progressively (up to 60 min) with a reduction of kinase concentration. In the subsequent Figures, unless stated otherwise, turkey gizzard kinase was used together with an excess of isolated L_{20} as a substrate. (b) Relationship between phosphorylation rate and substrate concentration at different MLCKase concentrations. As in (a) the specific activity increased with kinase concentration (given on each curve), but the relationships were hyperbolic for the lower range of the concentrations used. (This was judged from the linearity of the double-reciprocal replots.) At MLCKase concentrations above $1.0 \mu\text{M}$ (c) these v versus S relationships were not hyperbolic and exhibited positive co-operativity with n_H approaching 2. For this type of data the Hill equation (Segel, 1975) was used as the model function since kinase concentration can be considered as being negligible relative to that of L_{20} .

dependence of their specific activity on concentration. It therefore constitutes the major departure from the classical Michaelis-Menton behavior. Figure 1(a) shows that smooth muscle MLCKase belongs to this class of enzymes. In a typical experi-

ment, increasing amounts of MLCKase and CM (at 1 to 1.2 molar ratio) were added to a fixed concentration of substrate (purified L_{20}) and with the concentration of the latter held high to ensure V_m conditions (zero order kinetics). The specific activity

of the enzyme increased several fold over the relatively wide range of concentrations tested.

Allosteric effects can also be induced by a specific interaction of enzyme and substrate (Kurganov, 1982) and these manifest themselves as a non-hyperbolic relationship between v (rate) and S (substrate concentration). At relatively low MLCKase concentrations the observed relationship was hyperbolic (Fig. 1(b)) as judged from the linearity of the double-reciprocal replots, but allosteric effects were nevertheless present. As shown in Figure 1(b) the specific activity of MLCKase nearly doubled after a fourfold increase of the kinase concentration. Thus, the V_m and K_m were both dependent on the enzyme concentration even at low kinase concentrations. The specific activity also depended on the substrate concentrations and this was particularly clear when intact myosin was used as a substrate (Fig. 2(b)).

In the experiments shown in Figure 1 CM was present in molar concentrations that slightly exceeded those of MLCKase. The Ca^{2+} concentration was also maintained at a high enough level to ensure full saturation of all four Ca^{2+} binding sites so that the observed kinetic effects were solely due to the substrate and the enzyme interaction. At higher enzyme concentrations, but still negligible relative to the concentration of the light chain, the relationship between the rate and the substrate concentration exhibited some co-operativity (Fig. 1(c)). The value of the Hill's coefficient (n_H) approached 2, although a sigmoidal shape of the plot in Figure 1(c) was not obvious. The n_H coefficient of 2 relative to the substrate suggests that MLCKase is able to form tetramers that have negligible affinity for the substrate (Kurganov, 1982).

(ii) Activation by CM

Activation of kinase by CM was found to be highly co-operative. Since CM can be considered as an essential activator, the empirical co-operativity, relative to CM, can readily be measured by application of our fitting procedure (see Materials and Methods) together with the empirical Hill equation as derived by Segel (1975) for such a case:

$$V = \frac{V_{\text{app}} \times [\text{CM}]^{n_H}}{K_d^{n_H} + [\text{CM}]^{n_H}}, \quad (1)$$

where $V_{\text{app}} = V_m \times [S]/([S] + K_s)$. In most of the experiments of this type the substrate concentration ($[S]$) was kept at saturating levels, therefore $V_{\text{app}} \approx V_m$. The latter condition, however, does not affect the numeric procedure since only the parameters n_H , K_d and V_{app} were taken as the variables.

Using this formula, average co-operativity coefficients (n_H) between 3.5 and 4.5 were obtained irrespective of whether isolated L_{20} (Fig. 2(a)) or intact myosin (Fig. 2(b)) was used as a substrate. These values of Hill's coefficient were obtained relative to total, rather than free, CM concentration. Under normal experimental conditions the total kinase concentration ($[E]_t$) was lower than that of CM, but

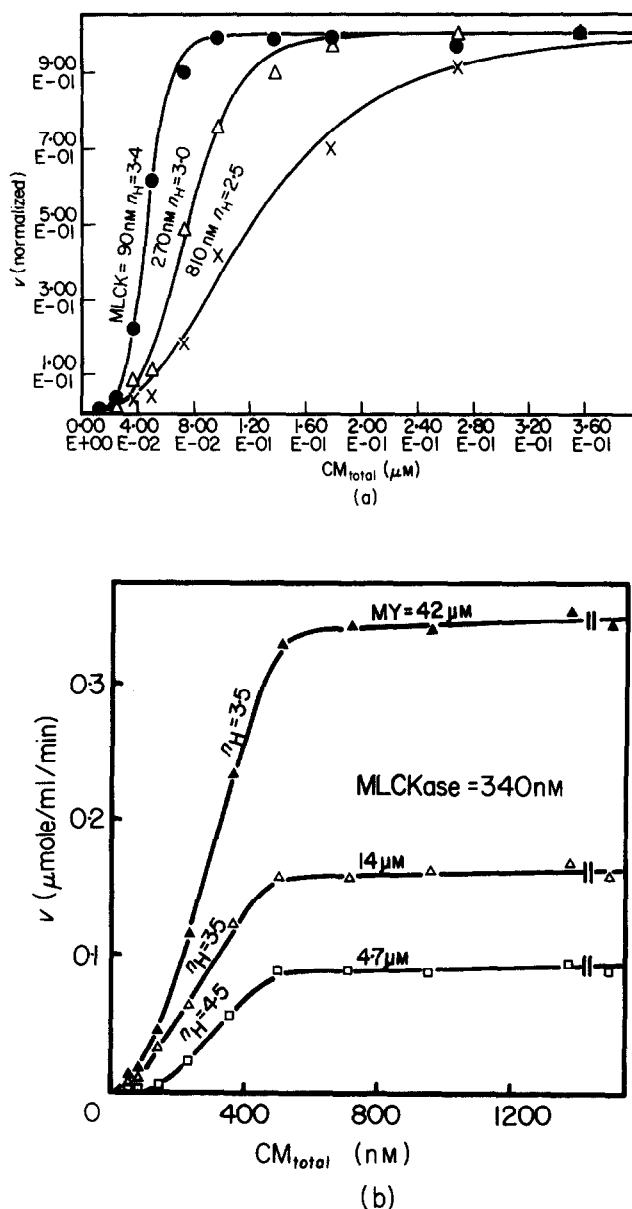


Figure 2. Co-operative activation of MLCKase by CM in the presence of saturating concentrations of Ca and with L_{20} (a) or intact myosin (b) as a substrate. (a) Continuous lines represent best computer fits using a modification of the empirical Hill equation derived in the present study. For more details see Results.

not negligible, therefore the CM fraction bound (to the kinase) should approximately be equal to $[E]_t \times v/V_m$ assuming that only one-to-one kinase-CM complexes are active. The free CM could then be estimated by the following correction: $[\text{CM}]_f = [\text{CM}]_t - [E]_t \times v/V_m$ (Edelman & Krebs, 1982). The application of this correction together with our fitting procedure gave even higher values of the empirical co-operativity coefficients (n_H). It is illustrated in Figure 3(a) (broken line) where the steepness of the CM activation curves increased after the correction while preserving their sigmoidal character. The higher corrected n_H values appeared to be

erroneous since the presence of non-catalytical concentrations of the kinase should introduce an opposite effect. (A "titration" of the enzyme introduces an apparent increase in co-operativity thus the correction should produce an apparent decrease in co-operativity.) In view of this another method of measurement of empirical co-operativity was sought in the present study. By analogy to a situation when, as for Michaelis-Menten kinetics, the enzyme concentration is high, the concentration of the CM/MLCKase complexes can be approximated by:

$$[ECM] = -\sqrt{\{([E]_t + [CM]_t + K_1)^2 - 4[E]_t \times [CM]_t\}} \quad (2)$$

(Segel, 1975) and the free calmodulin $[CM]_f = [CM]_t - [ECM]$. By introducing this expression into the Hill equation, a convenient model function was obtained for analysis of the empirical co-operativity of the experimental data. (From the numerous applications of this function to the experimental data it became clear that the apparent binding constants K_d and K_1 could not be assumed to be the same.) In contrast to the previous correction, the application of this model function provided n_H values that were somewhat lower than those obtained simply when the total CM concentration was used. Now, the co-operativity coefficients (n_H) were in the range 2.5 to 3.5 and required exact values for the concentration of the active sites which might not be equal to the kinase concentrations.

These high values of the Hill's coefficients could be explained in terms of allosteric effects. Since the saturation rate (maximal activity) appeared to be attained at approximately a one-to-one molar ratio of CM to kinase, the effects were not of a common type where a simple oligomerization of an enzyme results in a co-operative interaction between the protomers. Moreover, the question arose whether or not CM was directly responsible for the observed high degree of positive co-operativity.

(iii) Activation by CM at different Ca^{2+} levels

As described above, the CM-activation curves were co-operative (sigmoidal) at saturating levels of Ca^{2+} (Fig. 2(a)). However, as the concentration of Ca^{2+} was reduced or the range of CM concentration increased, the co-operativity and amplitude of the activation appeared to decrease (Fig. 3). Until now, this type of data has been analyzed by the Adair approach (1925). This approach provides a relationship between MLCKase rate and CM and Ca^{2+} concentrations. Thus, it can be used in evaluation of at least some of the required nine binding constants from the data of the type shown in Figure 3 (Huang & King, 1985). Applications of the rate relationship derived by Huang & King (1985) to these data in our minimization procedure (see Materials and Methods) indicated that this relationship was not adequate even at high CM concentrations. An analogous formula derived without rapid equilibrium conditions (unpublished results) described the kinetic data more satisfactorily

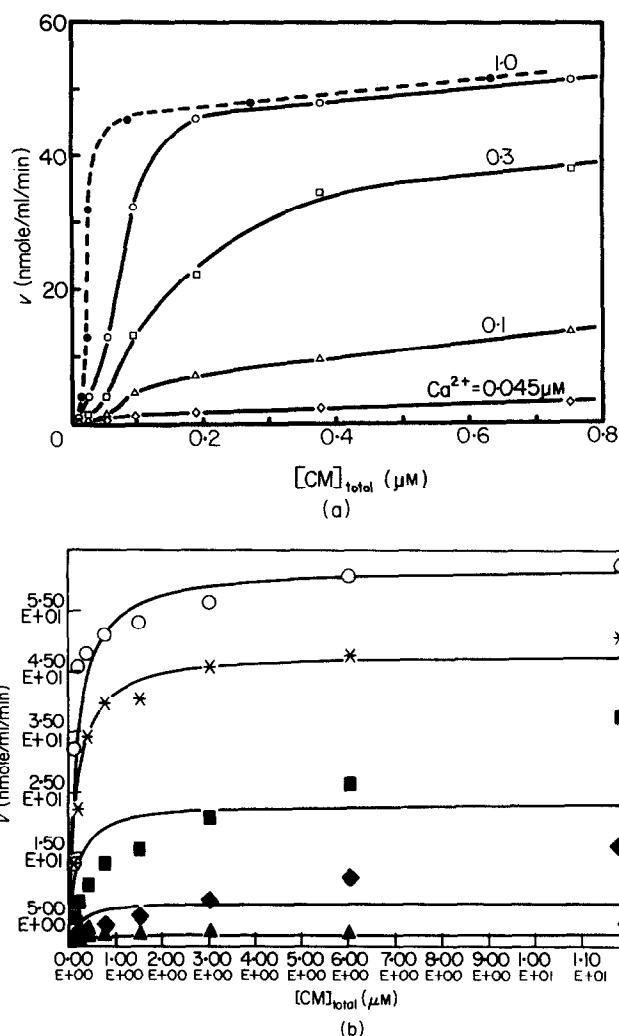


Figure 3. CM-activation of MLCKase at variable Ca^{2+} concentration (given above each activation curve). (a) The low and (b) the high range of CM concentrations. MLCKase concentration was 100 nM. In (a) only experimental curves are shown. The broken-line curve represents a correction of the uppermost curve (1.0 μ M) for CM bound to kinase as described by Edelman & Krebs (1982). In (b) continuous computer lines represent a best-fit obtained using a rate formula derived in the present studies (not presented) with the minimization software (see Materials and Methods).

(Fig. 3(b)) even though only the two constants (a corresponding geometric and an arithmetic average) were minimized. However, it was clear from this analysis that this rate relationship could not predict the positively co-operative activation by CM observed experimentally (see Fig. 3(a)). Thus, in general, the Adair approach cannot be applied for an analysis of this type of experimental data.

(iv) Dependence of MLCKase activity on its ratio to CM

Measurements were made of the phosphorylation rates as a function of CM to MLCKase ratio (Fig. 4). By increasing the amounts of kinase while decreasing the amounts of CM, a relatively wide

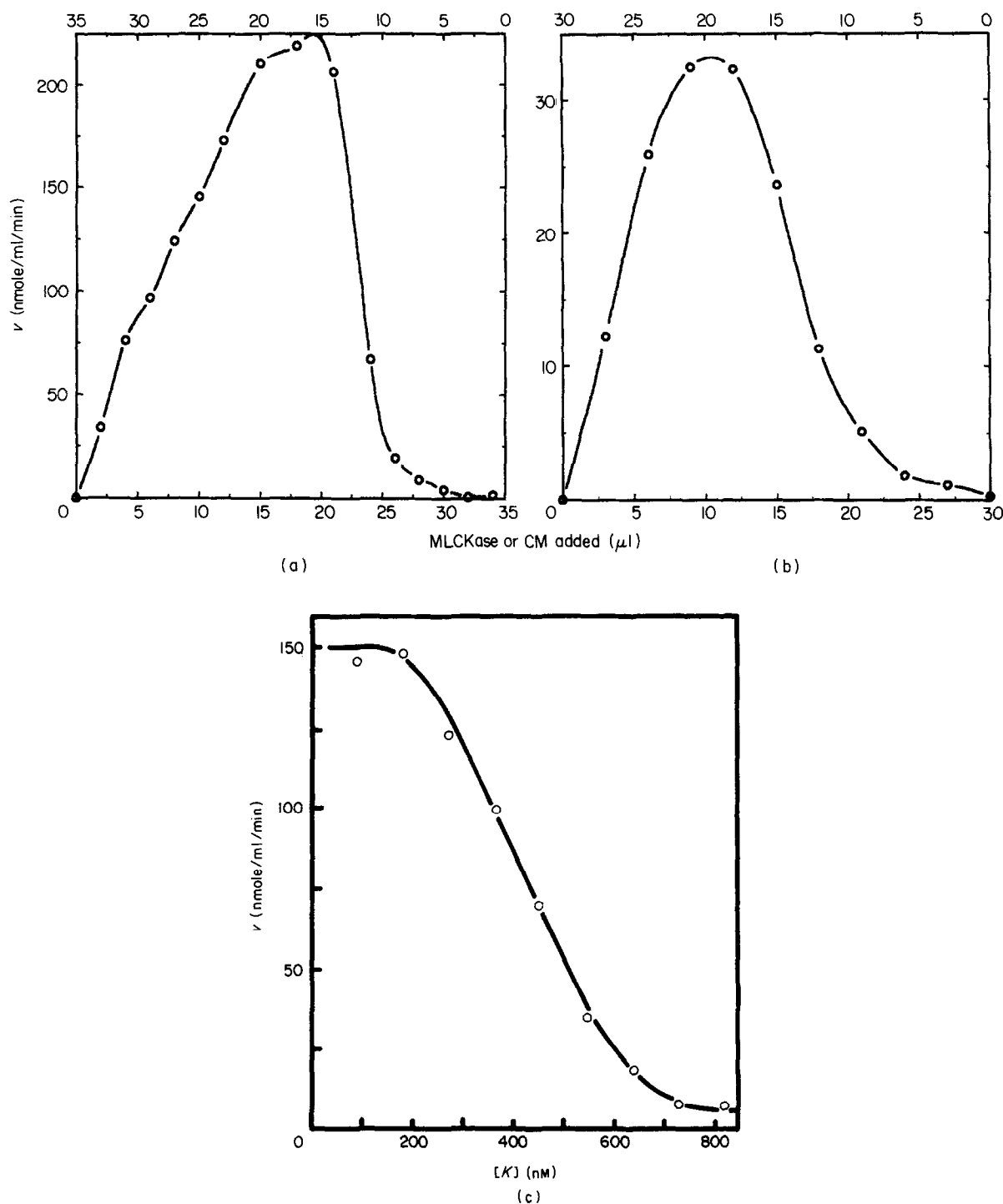


Figure 4. MLCKase activity as a function of its ratio to CM in the constant volume assays. Increasing amounts of kinase (lower axis) and decreasing amounts of CM (upper axis) were added and the phosphorylation rates measured. (a) and (b) The activity curves were not symmetrical indicating that CM did not act as a simple essential activator. The concentrations were (a) $5.0 \mu\text{M}$ and (b) $2.4 \mu\text{M}$ for kinase as well as (a) $3.0 \mu\text{M}$ and (b) $1.0 \mu\text{M}$ for CM. (c) When the activity was expressed per unit of the kinase concentration only the inhibition was apparent.

range of these ratios could be investigated in a constant assay volume. There was a striking decrease in MLCKase activity above a CM to kinase ratio of 1 : 1, the optimal stoichiometry. At ratios of CM to kinase of 1 : 4 there was complete inhibition of the enzyme activity. The activity plots shown in Figure 4(a) and (b) were not symmetrical. The rate

increases were linear on the left-hand sides and sigmoidal on the right-hand sides. Thus, activation of kinase by CM was not equivalent to the activation of CM by kinase, as would be expected for a simple essential activator (Reiner, 1969). The rate at constant CM and variable MLCKase concentrations was therefore measured (Fig. 5). These experi-

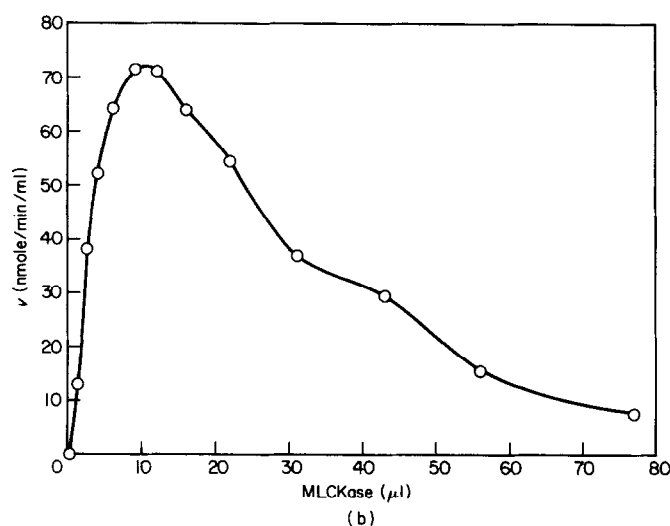
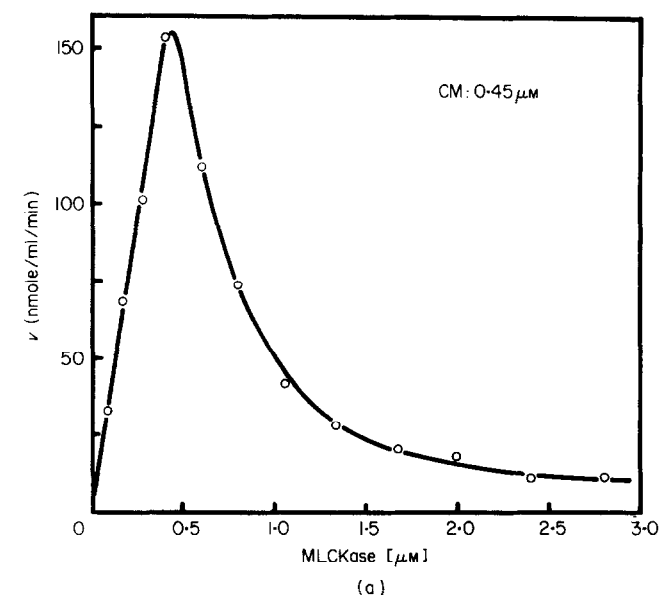


Figure 5. (a) Biphasic dependence of MLCKase activity on its concentration at a fixed CM concentration. Note that after reaching a maximum at an approx. 1 to 1 molar ratio of CM to kinase, the activity rapidly decreased and became negligible at a 4-fold or higher kinase concentration. (b) The plot was obtained using MLCKase from pig stomach muscle.

ments showed a clear biphasic dependence of rate on the kinase apoenzyme concentration (Fig. 5(a)). In the latter type of assays a much smaller range of MLCKase concentrations could be covered and, as a result, they required very appropriate concentrations of the reactants. Replots of both types of data, with the activity expressed per unit of MLCKase, produced a different type of relationship where the inhibition of the kinase was only apparent (Fig. 4(c)).

As is shown in Figure 5(b), purified pig stomach MLCKase exhibited exactly the same properties with respect to activation at constant CM and variable kinase concentrations. The only noticeable difference was that the inhibition was taking place

at a somewhat lower ratio of kinase to CM of about 3 to 1. However, this ratio was, in general, variable and was different for different types of MLCKase preparations.

A biphasic dependence of MLCKase activity such as shown in Figures 4 and 5 provided further experimental evidence for a co-operative activation of MLCKase relative to CM. From these experiments it was clear that at the lower and substoichiometrical range of CM concentrations association between CM and kinase did not result in enzyme activation, but at high CM to kinase levels enzyme activation was positively co-operative.

(b) Oligomerization of MLCKase

(i) Gel filtration experiments

One explanation for the behavior of MLCKase at low CM levels would be that kinase might have formed inactive oligomers. As the CM concentration was increased these could dissociate into active protomers. The equilibrium between the high (oligomeric) and the low (protomeric) relative molecular mass forms could depend on the total kinase concentrations as well as on its ratio to CM. To test this hypothesis the relative molecular mass of MLCKase was measured in the presence of different CM concentrations. MLCKase was applied to a standardized Sepharose 6B-CL column and its elution profile compared with that of other standard proteins of known relative molecular mass. As shown in Figure 6(a), the kinase peak comigrated with that of thyroglobulin having a relative molecular mass of 659×10^3 and eluted clearly before ferritin or catalase. Taking into consideration that the relative molecular mass of the gizzard kinase might be as low as 107×10^3 (Olson *et al.*, 1990) the kinase appeared to elute as a hexamer. If, however, the relative molecular mass is about 130×10^3 , as is generally accepted (Adelstein & Klee, 1981), then the position of the peak indicates a formation of tetramers. The kinase elution peak was relatively wide and its width was somewhat larger than that of other calibration proteins applied onto the same column at a similar protein concentration. This was particularly evident for the kinase peaks obtained from runs on a Sephaeryl S-300 column (data not presented). In the latter case the peaks were asymmetrical with a broadening at the high relative molecular mass side. This indicated that kinase was not present in a single oligomeric form. The application of kinase at fivefold-higher concentrations (or more) appeared to confirm this motion since, irrespective of the gel used, the kinase peak became even wider so that the high absorption was present in all separation tubes of the column. Such runs, in addition, commonly showed a void volume peak of even higher relative molecular mass range. In contrast, at the lowest optically detectable concentrations kinase commonly eluted at a much lower relative molecular mass range (Fig. 6(a)). Thus, it was clear that kinase formed oligomeric aggregates of various molecular sizes.

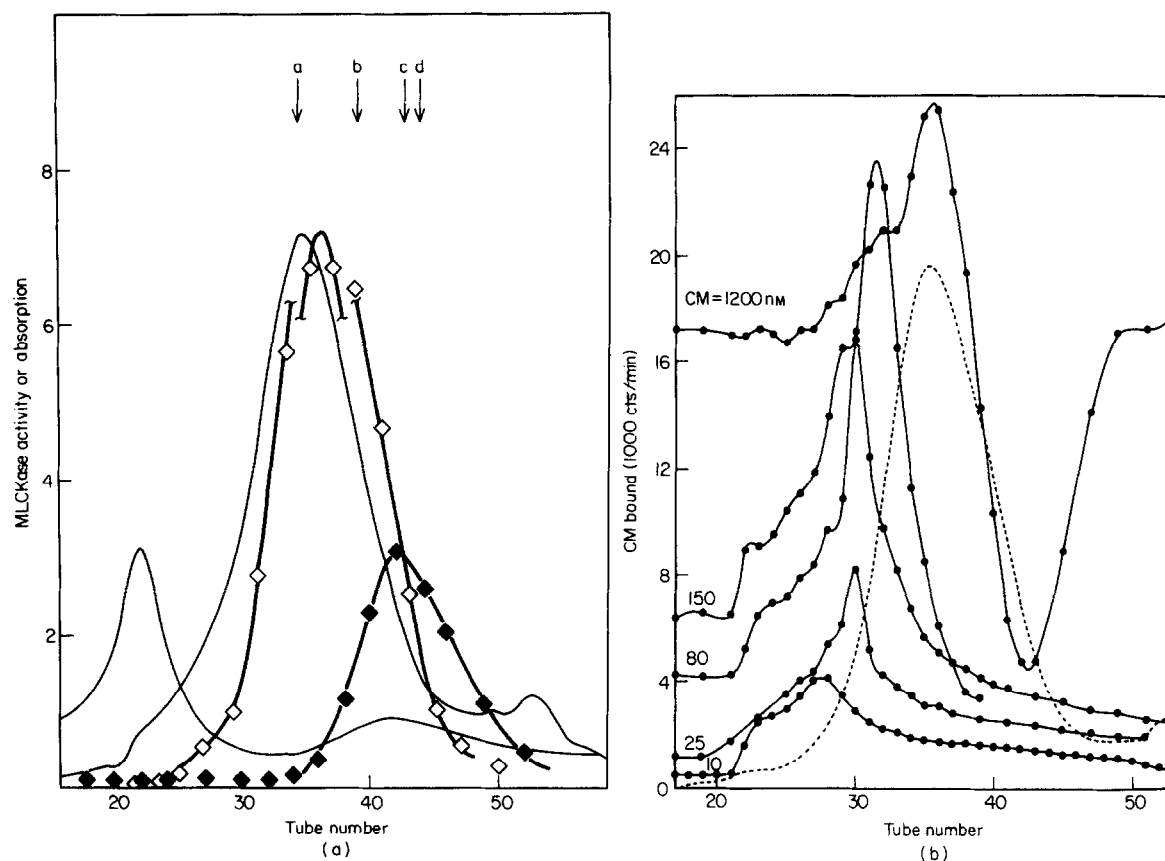


Figure 6. (a) Oligomeric size distribution of MLCKase as judged from its gel filtration profiles on the standardized Sepharose 6B-CL column. The major peak in the centre (continuous line) represented an absorption profile obtained at a standard kinase concentration ($14 \mu\text{M}$ in the sample). The kinase elution profile was very much the same as that of thyroglobulin having a relative molecular mass of 670×10^3 , but a corresponding MLCKase activity peak was located on its right (lower relative molecular mass) shoulder (\diamond). The kinase, at much lower concentration, eluted as a peak at a lower relative molecular mass range (\blacklozenge) with a correlating absorption peak (tubes 38 to 45). The void volume peak (tube 19 to 23), also seen in this run, was commonly observed and might correspond to the inactive, high relative molecular mass kinase oligomers. The activities and absorptions of kinase are comparable but they were plotted in arbitrary units. The positions of the maxima corresponding to the runs with the calibration proteins were marked with arrows; (a \rightarrow) thyroglobulin, (b \rightarrow) ferritin, (c \rightarrow) catalase and (d \rightarrow) aldolase. For more details see text. (b) Binding of CM by MLCKase during gel filtration experiments on the standardized Sepharose 6B-CL column. The column was equilibrated with increasing concentrations of [^{125}I]CM and, after application of the same volume of kinase (absorption peak, broken line), the distribution of bound CM measured. Note that as the CM concentration increased the peak corresponding to the maximal binding shifted gradually towards tubes containing lower relative molecular mass forms of kinase. CM concentration (nM) was given for each binding run. The curve corresponding to the highest CM concentration (1200 nM) should have a scale with about 10-fold larger range.

Fractions from the Sepharose 6B-CL column were also examined for MLCKase activity. These measurements demonstrated that the kinase activity peaks did not correlate very well with a corresponding protein peak. As it is clear from Figure 6(a) the maximal activity was observed at the low relative molecular mass shoulder of the latter peak (Fig. 6(a)), indicating that the MLCKase oligomers had reduced catalytic activity while the protomers, and possibly the dimers, were active. Addition of an excess of CM during MLCKase activity measurements did not result in a broadening of the kinase activity peak or an appearance of an additional peak. The distribution of kinase into its various oligomeric forms was therefore not governed by CM. This was particularly clear when kinase from tubes 34 to 44 of the Sepharose 6B-CL

column was reapplied onto the same column. (The kinase concentration in the first run had to be relatively high.) In this case the kinase eluted as two peaks; the first in the void volume (tubes 20 to 24) and the second as the kinase protomer (tubes 39 to 46) with the activity found only in the latter peak (Fig. 6(a)).

(ii) Binding of CM

More conclusive evidence for the absence of CM involvement in the oligomerization process came from experiments in which the same MLCKase preparation had been repeatedly applied together with different concentrations of added CM (data not presented). From these experiments it was obvious that neither the position nor the width of the kinase peak changed irrespective of CM concentration.

In another series of runs on the standardized Sepharose 6B-CL column, an apparent CM-binding affinity and stoichiometry of the various oligomeric forms of kinase was established using radioactively labeled CM (see Materials and Methods). It is clear from Figure 6(b) that as the concentration of free CM increased the radioactivity peak progressively shifted towards the kinase forms having an apparent smaller molecular size. Correspondingly, with the column equilibrated at 5 to 10 nM-[125 I]CM the binding was mainly observed in the tubes 21 to 30 (Fig. 6(b)) and kinase eluted in these tubes had apparently the highest affinity for CM. At 15 to 25 nM-free CM concentration the maximal binding appeared at the tubes 28 to 32, while at 50 to 100 nM-CM, the position of the peak shifted to tubes 33 to 36. The binding peak corresponding to the MLCKase absorption peak occurred at above 0.5 μ M of the CM concentration but its binding stoichiometry greatly exceeded that of the kinase (Fig. 6(b)).

4. Discussion

(a) Allosteric properties of MLCKase

The demonstrated allosteric effects of MLCKase were as follows. (1) An increase in the affinity of CM for Ca after formation of the CM-MLCKase complex resulting in co-operative activation of kinase relative to Ca; (2) dependence of the specific activity of the CM-MLCKase complex on its concentration; (3) the substrate induced effects i.e. a co-operative activation by the substrate as well as a dependence of the specific activity on the substrate concentration and (4) oligomerization of kinase into high relative molecular mass complexes with negligible (isolated L₂₀) or twofold lower (intact myosin) specific activities relative to the activities exhibited by kinase dimers or monomers.

These kinetic properties of MLCKase correspond to those listed by Kurganov (1982) as characteristic of an allosteric enzyme. The time-dependent kinetic effects (e.g. lag periods or initial burst) that were not detected in the present study may represent an exception. However, the time-scale investigated was in the range of several seconds and the existence of faster or considerably slower effects of this type cannot be excluded.

From the four allosteric effects listed above, the first has already been described for skeletal (Olwin *et al.*, 1984) as well as for smooth (Mamar-Bachi & Cox, 1987) muscle MLCKase. It has been explained in terms of energy-coupling after attachment of CM to kinase, resulting in a strong increase in the affinity of at least some of the Ca-binding sites on CM. As a result there is a co-operative activation relative to Ca. The second effect, although not previously demonstrated, represents a very general property of all allosteric enzymes and at the same time bears no indications of a possible mechanism involved. According to Kurganov (1982), the third effect indicates that an allosteric enzyme forms tetramers

(here oligomers) that have much lower affinity for the substrate than the monomeric (or dimeric) forms of the enzyme. Both the latter effects have not been demonstrated previously, possibly because all studies employed relatively low concentrations of MLCKase (Malencik & Anderson, 1986). The fourth effect was MLCKase oligomerization and this is considered below. In general, although the present studies offer no possible mechanistic explanations, the findings have significant implications for the kinetic behavior of the enzyme.

(b) Oligomerization

The most interesting aspect of the allosteric properties of MLCKase described here is, no doubt, its oligomerization (most likely tetramerization). The significant consequence of this oligomerization was a co-operative activation of kinase by CM that has not so far been demonstrated. Thus, the binding of MLCKase by CM appeared to be symmetrical in the sense that each of the four Ca-binding loops was able to bind one kinase molecule. It remains to be seen how this relates to the tertiary structure of CM, which has recently been described by Babu *et al.* (1985, 1988). One important aspect of the interactions within such an oligomer complex was that the affinity for CM increased as the size of the complex increased. Independently, CM was not observed to be directly involved in the oligomerization process and the equilibrium among the various oligomeric species was governed by other factors that remain to be established. There are some preliminary indications that they may include a direct covalent cross-linking of kinase monomers or autophosphorylation-dependent affinity modification (unpublished results).

Following the elucidation of the three-dimensional structure of CM (Babu *et al.*, 1985, 1988), the mechanism of MLCKase regulation was intensively investigated (Means & George, 1988). It has now been shown that the active-site of MLCKase is blocked as a result of the amino-terminal portion of the molecule having a strong sequence homology to its substrate. CM acts as a derepressor rather than a direct activator of kinase by unblocking this site on binding. The formation of inactive oligomers indicates not only that binding of CM without unblocking may be possible but also that there is a strong affinity of MLCKase for itself. I suggest that the substrate homologous domain of kinase is responsible for this high affinity. Autophosphorylation of skeletal (Geuss *et al.*, 1985) as well as smooth (unpublished results) muscle kinases, taking place even at extremely low concentrations, clearly demonstrates that a kinase molecule can bind to another with a very high affinity.

Very recently it has been suggested that allosteric-type regulation might be a general property of most of the CM-dependent enzymes (Jarrett & Madhavan, 1991). This prediction is based on detection of a melittin (CM-like) binding site in the regulatory binding site of these enzymes, in addition

to the known CM-binding site. The model proposed by Jarrett & Madhavan predicts a CM-independent oligomerization of the enzymes and is therefore consistent with the experimental data from the present studies. A similar self-association has also been demonstrated for another well-known CM-dependent enzyme, namely plasma membrane Ca^{2+} -ATPase (Vorherr *et al.*, 1991). As expected from the model, the CM-binding domain of this latter enzyme was shown to be involved in this process.

From the oligomeric properties of MLCKase described here, it appears that kinase regulation is not similar to that of phosphofructokinase, for which only some of the allosteric properties deriving from its interaction with CM have been described (Mayr & Heilmeyer, 1983; Mayr, 1984). Perhaps there are more similarities to another widely known Ca/CM-dependent enzyme, namely erythrocyte Ca^{2+} -ATPase. The activity of the latter enzyme is regulated by oligomerization although, as for MLCKase, CM itself is not directly involved in this oligomerization process (Kosk-Kosicka & Bzdega, 1990; Kosk-Kosicka *et al.*, 1990).

(c) MLCKase and contraction

The data presented here represent a first indication that oligomerization and Ca/CM-induced allosteric effects might be involved in the regulation of smooth muscle MLCKase activity. Therefore, in smooth muscle contraction CM not only acts as an exclusive essential activator but also as an allosteric regulator of kinetic activity. Although the exact mechanism of such a regulation has not been deduced, some of its physiologically relevant characteristics were described here. Surprisingly few Ca/CM-dependent enzymes of this type are described in the literature and this aspect of allosteric regulation requires extensive experimental work in the future as is clear from a recent monograph by Kurganov (1982). More data have been accumulated on allosteric regulation involving interconvertible enzyme cascade systems (Chock & Stadtman, 1980). The author considers that the latter type system controls contraction of smooth muscle. In this case, and as has been suggested previously (Sobieszek, 1985a), myosin phosphorylation/dephosphorylation cycle together with its corresponding enzymes (MLCKase and MLCPase) may represent the first regulatory loop. The second loop would then include factors controlling activity of MLCKase such as autophosphorylation and a phosphatase dephosphorylating MLCKase. CM would then, also in the second loop, play the role of an allosteric activator. Studies are in progress to investigate the possible involvement of CM at different levels of the regulation of smooth muscle contraction.

I wish to thank Professor J. Karn for improving the text of the manuscript as well as Drs J.V. Small and

Simon Rea for helpful comments. The technical assistance of Ms U. Müller is also acknowledged.

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Edited by J. Karn

Erratum

Regulation of Smooth Muscle Myosin Light Chain Allosteric Effects and Co-operative Activation by Calmodulin

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J. Mol. Biol. (1991), **220**, 947–957

Formula (2) has been incorrectly printed. The first item of the formula was omitted and the correct formula is as follows:

$$[ECM] = \frac{1}{2}([E]_t + [CM]_t + K_1) - \sqrt{\{([E]_t + [CM]_t + K_1)^2 - 4[E]_t \times [CM]_t\}}.$$