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Oligomerization of Smooth Muscle Myosin Light Chain Kinase and Its Modifications by Melittin and Calmodulin

Abstract: *The catalytic activity of smooth muscle myosin light chain kinase (MLCKase) requires the presence of calcium and calmodulin* [*CM; J. T. Stull et al. (1993)* Molecular and Cellular Biochemistry, *Vols. 127/128, pp. 229–237*] *and can also be modified through its own oligomerization* [*E. B. Babiychuk et al. (1995)* Biochemistry, *Vol. 34, pp. 6366–6372*]. *In the present report we demonstrate that melittin, one of the most potent CM antagonists, interacted reversibly with the MLCKase apoenzyme with affinities comparable to those of CM and influenced the oligomeric state of the kinase. At low melittin to kinase ratios the kinase formed insoluble oligomers (aggregates) while at higher melittin concentrations it existed predominantly as soluble oligomers revealed by cross-linking as octamers and hexamers. The kinase alone exhibited similar biphasic solubility within a 5–30 μM range and its solubility was strongly influenced by the ionic strength of the medium. Melittin was also found to promote both the aggregation of the purified 24-kDa C-terminal fragment of the kinase and its analogue telokin, as well as of myosin light chains, but had no effect on the solubility of bovine serum albumin, caldesmon, or calmodulin. These data and our cross-linkage experiments indicate that the insoluble kinase oligomers arose via melittin-induced aggregation of the kinase dimers in which the telokin-like domain played a main role. The soluble oligomers, in turn, were formed after saturation of the kinase with melittin, which resulted in a weakening of the interaction between the protomers with an increase of the long-range order within the oligomers. This interpretation is consistent with the observed effects of melittin on MLCKase catalytic and autocatalytic activities. At concentrations of melittin required to produce soluble oligomers, the binding of the kinase to myosin filaments was considerably enhanced. A plausible mechanism for the formation of the soluble oligomers and aggregates is suggested and its relation to the possible MLCKase assemblies discussed in terms of a model.* © *1997 John Wiley & Sons, Inc. Biopoly* ***42:*** *673–686, 1997*

Keywords: *melittin; calmodulin; myosin light chain kinase; oligomerization; smooth muscle*

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INTRODUCTION

Myosin light chain kinase (MLCKase) is a calmodulin (CM) dependent enzyme responsible for the regulation of actin–myosin interaction in smooth muscle.[1,2] The kinase, fully inactive in the absence of Ca^{2+}/CM, binds to CM in response to an increase in myoplasmic Ca^{2+} concentration and activates the actomyosin system by phosphorylating the myosin regulatory light chains.

Like most of the calmodulin-activated enzymes, the kinase possesses a catalytic core and a regulatory domain that includes the calmodulin-binding site.[3] In the absence of CM, the regulatory domain inhibits catalysis by blocking the kinase active site within the core. The blocking or inhibition has been proposed to result from interaction between a pseudosubstrate autoinhibitory domain (homologous to the phosphorylation site on the myosin regulatory light chain) and the substrate binding site located within the catalytic core.[4,5] Binding of CM to the regulatory domain activates the kinase by removal of this inhibition. This simple intrasteric regulatory model, however, has been challenged by more recent observations that have shown that mutant CMs can bind to but fail to activate the kinase.[6] This indicates that besides the autoinhibition, an additional conformational change may be necessary for formation of the active MLCKase/CM complex. In addition, we have recently demonstrated that the activation of MLCKase by CM may also be modulated by oligomeric-type modifications.[7,8]

Activities of CM-dependent enzymes are greatly affected by a number of small peptides that are bound by CM in a Ca^{2+}-dependent manner (for a review see Ref. 9). These peptides are commonly used as probes for interaction between CM and its target enzymes. Among them a cytoactive peptide from bee venom, melittin, exhibits exceptionally high affinity for CM that is equal to or even higher than that of the CM-dependent enzymes themselves.[10,11] While these CM antagonists have served as important tools for studies of Ca^{2+}/CM-dependent pathways, the specificity of their interaction with CM remains unclear since they may also interact with the enzymes and/or their substrates.[9,12] In the present report we investigated in detail the interaction between smooth muscle MLCKase and melittin. Interestingly, melittin was found to have pronounced effects on the oligomeric state of the kinase apoenzyme and its affinity of binding to myosin filaments.

EXPERIMENTAL PROCEDURES

Proteins

Myosin, MLCKase, and calmodulin were purified from turkey gizzard as previously described.[13,14] Myosin regulatory ($ReLC_{20}$) and essential ($EsLC_{17}$) light chains were isolated by the method of Perrie and Perry[15] and purified by gel filtration and ion exchange chromatography.[16] Telokin was prepared according to a procedure developed in our laboratory.[17] Turkey gizzard caldesmon was purified essentially as described by Bretscher[18] using an actomyosin preparation as starting material. Concentrations of the light chains, telokin, caldesmon, and bovine serum albumin were measured by the Biuret method,[19] while that of MLCKase and CM were measured from their absorption at 278 nm using extinction coefficients of $E^{0.1\%} = 1.1$ for the kinase[20] and $E^{0.1\%} = 0.18$ for CM.[21]

Melittin (cat. no. M2272, Sigma Chemical Co., St. Louis, MO) was further purified on a Sephadex G-50 gel filtration column followed by cation exchange chromatography on a CM-Sepharose column. CNBr-activated Sepharose-4B was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) and cross-linked with the ligand as recommended by the manufacturer. All chemicals used were of analytical grade and were purchased either from MERCK (Darmstadt, Germany) or from Fluka Chemie AG (Buchs, Switzerland).

Turbidity Measurements

Unless otherwise stated, concentrated stocks of melittin (1.1 m*M*), calmodulin (600 μM), or NaCl (4*M*) were added, in small aliquots (2 μL), to 400 μL solutions of MLCKase or other proteins tested—all at 5 μM. After mixing (1 min) turbidity was measured at 400 nm using a Beckman DU-60 spectrophotometer. The composition of the assay buffer (AA) used throughout the study was as follows (in m*M*): KCl—60; $MgCl_2$—2; dithioerythritol—0.5; and imidazole—10. The pH was adjusted to 7.5 at 4°C. When the assays were performed in the presence of CM, $CaCl_2$ was added to the solution to a final concentration of 0.1 m*M*.

Analysis of the Kinase Oligomerization

Insoluble, fine aggregates were pelleted using a small table centrifuge at 13,000 rpm for 30 min (low speed centrifugation). Sedimentation of the soluble oligomers required centrifugation at top speed in a Beckman airfuge for 30 min (high speed centrifugation). To remove traces of the supernatant, the tubes containing the pellets were first rinsed with water, and then the pellets were dissolved in 5*M* urea. Samples of the supernatants and pellets were analyzed by 7–18% gradient sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE).[22] MLCKase oligomerization was also analyzed by zero-length cross-linking with 5 m*M* 1-ethyl-3(dimethylami-

nopropyl)carbodiimide hydrochloride (EDC)[8] and by standard electron microscopy using carbon grids stained with uranyl acetate. During these experiments the kinase concentration was 10 μM.

Trypsin Hydrolysis of MLCKase

The kinase (10 μM) was hydrolyzed with trypsin at a kinase to trypsin ratio of 40:1 (w/w) for 30 min at 25°C. The hydrolysis was terminated by adding a 2-fold excess of soya bean inhibitor or by boiling the samples together with added ''SDS-mix.''[22] Individual tryptic fragments of the kinase were purified essentially as described by Ikebe et al.[23]

Phosphorylation and Autophosphorylation Measurements

MLCKase catalytic activity measurements were carried out at the highest possible MLCKase concentrations (2.5 μM) corresponding to the conditions favorable for oligomerization. This required the use of short assay times (15 s) and a low assay temperature (4°C). The concentrations of added Ca^{2+} and CM were respectively 0.1 mM and 5 μM. Other conditions were the same as previously described.[13,14] The activities of the 61 kDa fragment (2.5 μM) were measured without added CM.

Autophosphorylation rate measurements were carried out at 12 μM concentration of the kinase for 10 min. For the intermolecular autophosphorylation, CM (6 μM) and $CaCl_2$ (0.1 mM) were added while for the intramolecular autophosphorylation 2 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′,-tetraacetic acid (EGTA) was present. Other procedures were as described elsewhere.[24]

Binding of MLCKase to Myosin Filaments

Binding of the kinase to myosin filaments was estimated using a sedimentation assay together with SDS-PAGE. Increasing concentrations of filamentous myosin (in a fixed volume) were added to kinase solution (5 μM) and, after 10 min preincubation at 25°C, the samples were centrifuged at 10,000× g for 30 min. The pellets obtained were dissolved in 5M urea and analyzed, together with the supernatants, by SDS-PAGE.

RESULTS

Effects of Melittin on Solubility of MLCKase and Other Proteins

As demonstrated by absorption measurements at 400 nm (Figure 1), MLCKase formed very fine light-scattering aggregates in the presence of melittin. The turbidity was maximal after 2 min and remained constant for at least another 60 min. A similar aggregating effect of melittin was observed for telokin (Figure 2), an independently expressed smooth muscle protein that is identical to the C-terminal domain of MLCKase.[25] In agreement with observations of Malencik and Anderson,[26] no aggregation of bovine serum albumin was observed even at 250 μM of melittin (Figure 2). Likewise, addition of melittin to CM, or to caldesmon, produced no apparent modification of their solubility (Figure 2). In contrast and as previously reported,[26] melittin interacted with both the regulatory ($ReLC_{20}$) and essential ($EsLC_{17}$) light chains of smooth muscle myosin, causing their aggregation (Figure 2). The aggregation curves obtained for the light chains and telokin were sigmoid, indicating a high degree of cooperativity. This was confirmed by a nonlinear regression analysis of the aggregation curves using a modified Hill's formula.[14] The cooperativity coefficients obtained were between 2 and 3 for telokin and for the light chains with corresponding binding constants in the 2–3 μM range.

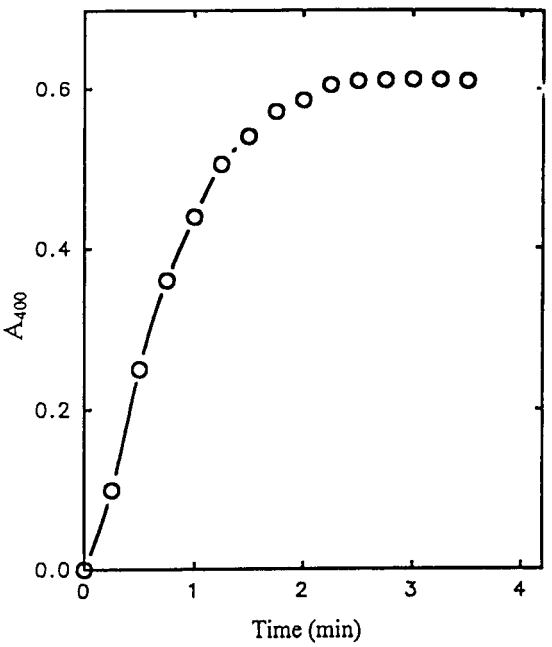

FIGURE 1 Aggregation of MLCKase in the presence of melittin. Melittin (5.5 μM final concentration) was added to clear MLCKase solution (5 μM) and, after mixing, the turbidity was measured at 400 nm as a function of time.

Biphasic Interaction of Melittin with MLCKase

The interaction of melittin with MLCKase was more complex than that with telokin or with the light

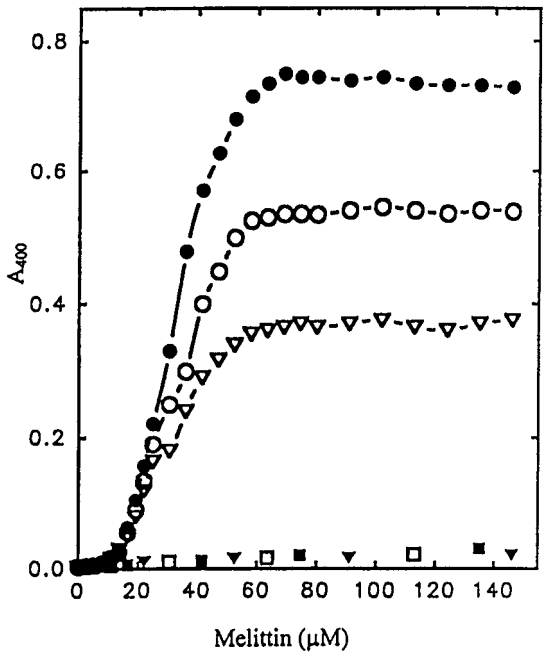

FIGURE 2 Effect of melittin on aggregation properties of selected proteins. The turbidities of the protein solutions (5 μM final concentration) were measured as function of added melittin. Note that increasing concentrations of melittin resulted in cooperative (sigmoid) aggregation of telokin (●-●), $ReLC_{20}$ (○-○), and $EsLC_{17}$ (▽-▽), but had no effect on solubility of bovine serum albumin (▼-▼), calmodulin (□-□), or caldesmon (■-■).

chains because it was biphasic (compare Figures 2 and 3). At relatively low concentrations of melittin, the kinase aggregation was comparable to that of myosin light chains and telokin (Figure 2), but when the melittin concentrations exceeded that of the kinase by 2-fold or more, there was a turbidity decrease and a consequent clearing of the MLCKase solution (Figure 3).

There was no simple correlation between the melittin concentration required for maximal aggregation of the kinase and the concentration of the kinase present (Figure 3). For the highest kinase concentration used (10 μM) an optimum was observed at a 2 to 1 molar ratio of kinase to melittin, while at 1 μM kinase an 8-fold excess of melittin was required (Figure 3). This type of behavior could be explained by relatively high affinity binding of melittin to multiple binding sites on the kinase with the K_d values in the range of 0.1–1 μM. No differences were observed in the effects of melittin in the presence or absence of Ca^{2+}.

Melittin is known to be bound by CM with an exceptionally high affinity (e.g., less than 0.1 n*M;* see Ref. 11). Because CM also binds strongly to MLCKase, we investigated the effects of CM on the MLCKase–melittin interaction. Addition of increasing concentrations of CM to a clear kinase–melittin solution (high melittin to kinase ratio) produced an increase and then a decrease in the turbidity (Figure 4). Thus, the effect of melittin could be fully reversed by CM as a result of direct depletion of the melittin by CM. This indicates that the affinity of melittin for calmodulin was higher than for the kinase. However, the affinity of melittin for the MLCKase apoenzyme was also in the nanomolar range because at least 6 melittin molecules remained bound to one kinase molecule even after gel filtration followed by exhaustive dialysis (Figure 4; inset).

Modification of the Oligomeric State of MLCKase by Melittin

In addition to the turbidity measurements, the kinase–melittin interactions were analyzed by sedimentation. As illustrated in Figure 5A, the amount of the kinase pelleted by low speed centrifugation, and therefore present in the aggregated state, increased in proportion to the concentration of melit-

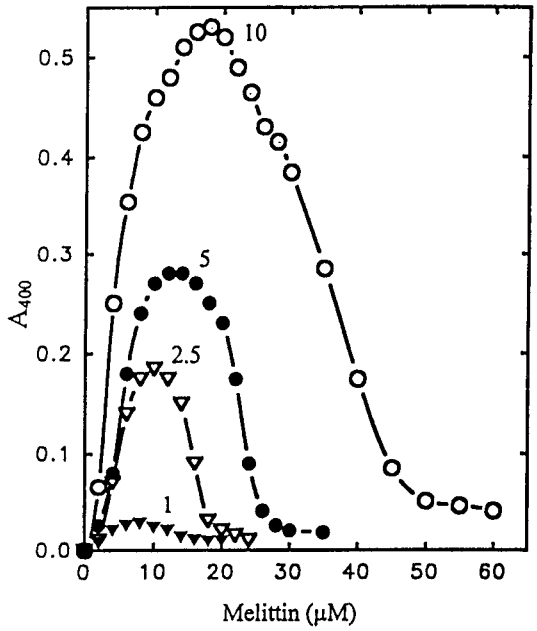

FIGURE 3 Biphasic solubility of MLCKase in the presence of melittin. The turbidity of MLCKase solutions was measured as a function of added melittin. The MLCKase concentrations (in μM) are given for each curve. Note that the turbidity increased steeply at low, and decreased at high concentrations of melittin.

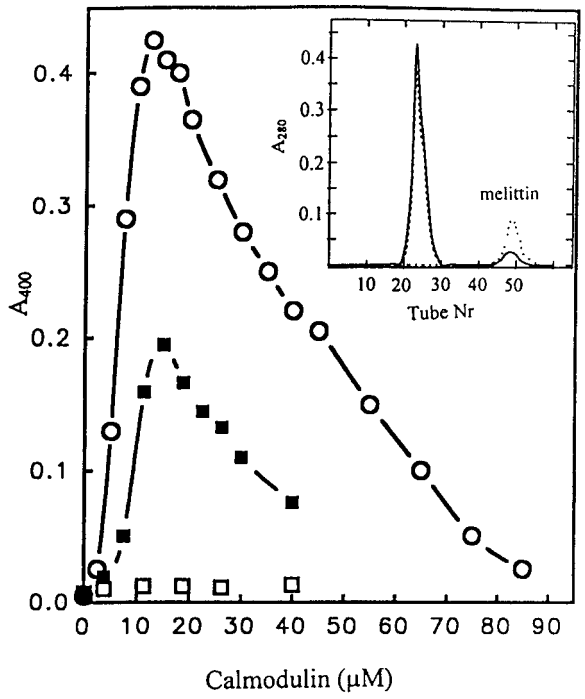

FIGURE 4 Effect of calmodulin on solubility of the melittin–MLCKase complex. Increasing concentrations of CM were added to soluble MLCKase–melittin complexes (5 and 40 μM, respectively) and turbidity measured. Note that the complex was insoluble at low CM concentration and then became soluble upon saturation with CM (○ - ○). After gel filtration of the same complex on a 0.6 × 100 cm Sephacryl S-100 column, 60% ammonium sulfate precipitation, and exhaustive dialysis (final concentration of the kinase was 2.5 μM), properties of the complex did not change (■ - ■). A control experiment for the kinase alone subjected to the same procedure (□ - □) is also shown. Inset shows the elution profiles of the kinase–melittin complex (solid line), kinase alone (dashed line), and melittin alone (dotted line). Note a 3-fold decrease of the melittin peak area due to its binding to kinase (compare solid and dotted lines) that was used to estimate the binding stoichiometry.

tin added, reaching a maximum between 20 and 30 μM of melittin. In agreement with the turbidity measurements, a further increase in the melittin concentration resulted in the solubilization of the kinase and, correspondingly, this form of the kinase remained in the supernatants during the low speed centrifugation (Figure 5A). This soluble kinase could, however, be pelleted by a high speed centrifugation (Figure 5B), indicating that it was in the form of soluble high molecular weight oligomers.

The effect of CM on the kinase–melittin interaction was also analyzed by sedimentation. For the soluble oligomers formed in the presence of 50 μM melittin (Figure 6, lines marked ''0''), the addition of relatively low concentrations of CM resulted in formation of the insoluble oligomers (Figure 6A). When CM concentration was high, most of the kinase remained in the supernatants even after high speed centrifugation (Figure 6B).

Formation of the insoluble and the soluble oligomers in the presence of melittin strongly depended on the ionic strength of the media (Figure 7), indicating ionic nature of the interactions. At very low salt concentrations (Figure 7A) the insoluble oligo-

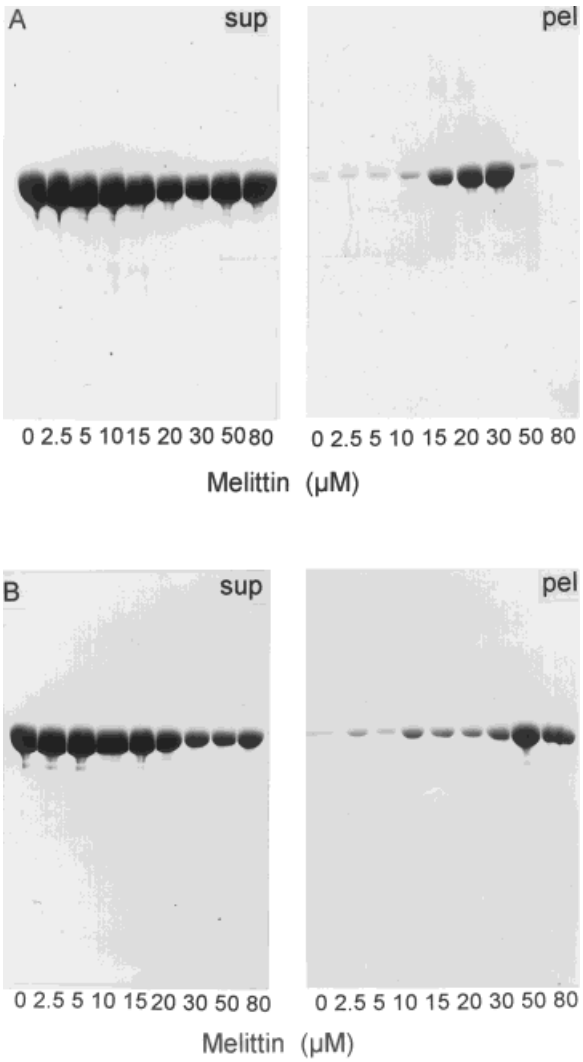

FIGURE 5 Sedimentation properties of MLCKase in the presence of melittin. MLCKase solutions (10 μM) were centrifugated either at 10,000× g (A) or 170,000× g (B) in the presence of indicated melittin concentrations. As expected from the solubilizing effect of melittin, no kinase sedimented in (A) at 50 and 80 μM of melittin. This soluble kinase was, however, pelleted by high speed centrifugation (B).

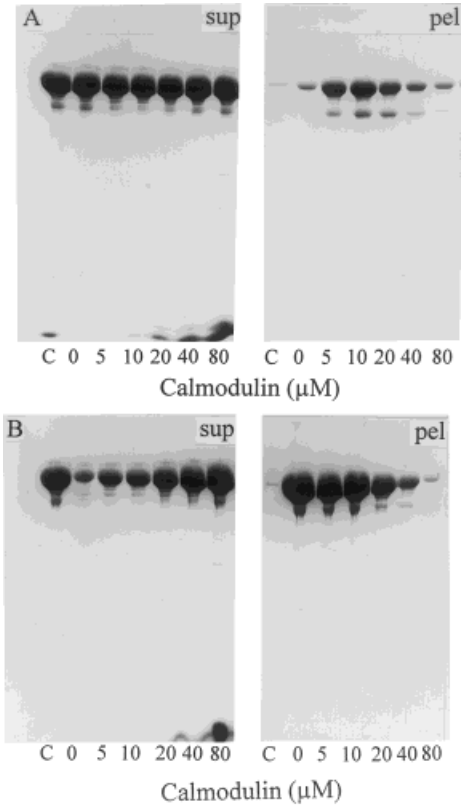

FIGURE 6 Sedimentation properties of melittin-induced MLCKase oligomers in the presence of CM. Condition were the same as in Figure 5(A and B) except the kinase and melittin concentrations (10 and 50 μM, respectively) and added increasing concentrations of CM. Controls (kinase–CM complex alone) are shown in the lines marked ''C.'' In agreement with the turbidity data, CM fully reversed effects of melittin on the kinase solubility.

mers were mainly formed, while 60 m*M* NaCl (Figure 7B) were optimal for formation of the soluble oligomers. Significantly, the latter were still present at physiologically relevant 120 m*M* NaCl, the conditions under which no insoluble oligomers could be detected (Figure 7A).

More detailed characterization of the oligomeric forms of the kinase was revealed by use of zero-length covalent cross-linking.[8] As shown in Figure 8, the presence of melittin during the cross-linking resulted in a decrease in the relative concentration of the kinase dimers; at the same time, the relative content of the kinase oligomers increased. As is apparent from the figure at intermediate melittin concentration there were, beside the monomers, relatively high concentrations of the dimers and relatively low concentrations of the oligomers. This indicates that the dimers, possessing a lower net charge, were neutralized by melittin resulting in their unspecific aggregation and precipitation. The hexamers and octamers seen on our SDS-PAGE patterns represent a minimal size of the oligomers as visualized by the cross-linking. The native oligomers, in solution, can be even larger (see below).

The MLCKase oligomeric forms were also examined by electron microscopy after negative staining. We could not detect any ordered assemblies of the kinase in the form of the insoluble oligomers. As expected, there were only large nonspecific aggregates present (data not shown). In contrast, the soluble oligomers formed in the presence of high melittin showed square-shape structures of surprisingly constant dimension (Figure 9). The size of these structures was in a range 24–36 nm, consistent with particles of 2 MDa molecular mass if one compares published electron micrographs of large enzyme assemblies (e.g., see Ref. 27). Thus, they could include 8 or more MLCKase dimers and their mode of assembly is considered in Discussion. The background between the oligomers most likely arose from the monomeric kinase, which amounted to a substantial part of the protein present (e.g., see Figures 5 and 6).

Oligomerization of Proteolytically Degraded MLCKase

Proteolysis of the kinase under conditions known to produce the 64, 61, and 24 kDa fragments[23] dramatically changed its response to melittin. At low melittin to kinase ratios both the native and hydrolyzed kinase preparations behaved similarly, forming insoluble oligomers (Figures 3 and 10A). However, at high melittin concentrations the characteristic biphasic response was not observed for the degraded kinase (Figure 10A). This indicates that the sites responsible for MLCKase oligomerization were cleaved away during the proteolysis. Of the three fragments present in the kinase digests, the 24 kDa species interacted with melittin with the highest relative affinity, as shown by binding to the melittin affinity gel (Figure 10B). With melittin added, this fragment exhibited similar solubility patterns to those of the hydrolyzed kinase or telokin (compare Figures 2 and 10A). In contrast, the 61 and 64 kDa kinase fragments did not aggregate upon addition of melittin. Thus, we concluded that the 24 kDa fragment was responsible for the formation of the insoluble oligomers.

Involvement of the 24 kDa fragment in the kinase oligomerization was also apparent from experiments in which melittin was added to the mixtures

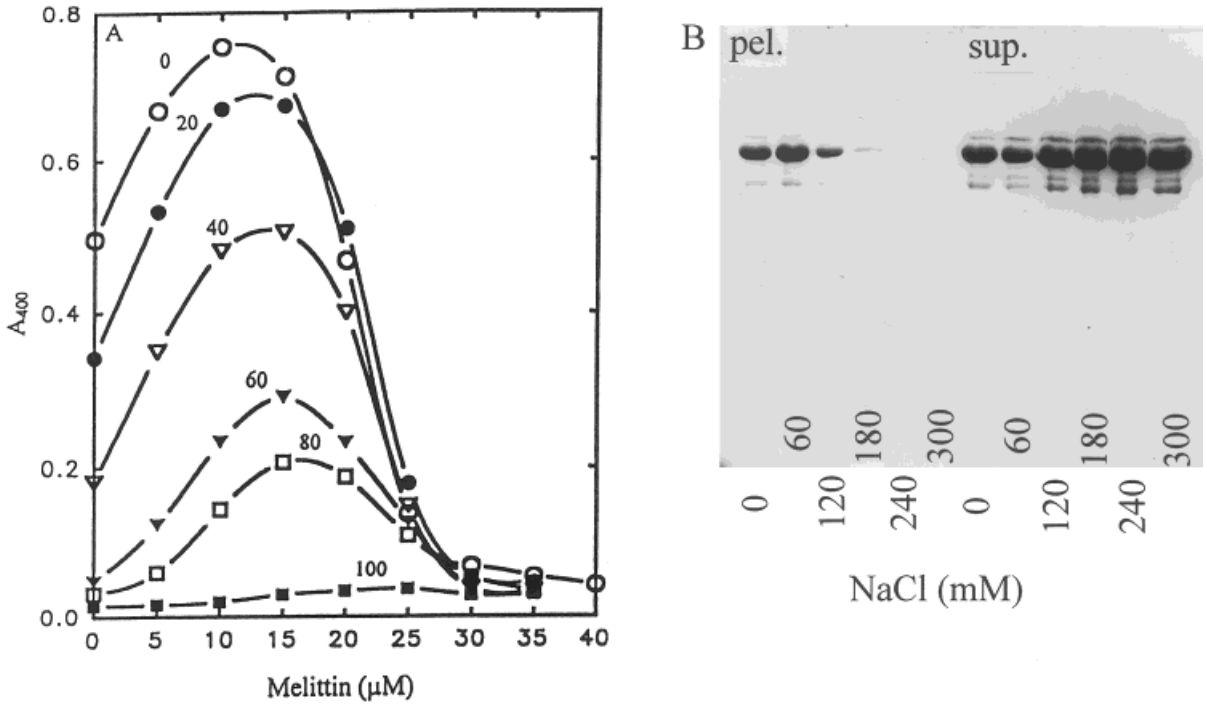

FIGURE 7 Effect of ionic strength on solubility of the kinase and the kinase/melittin complexes. In (A) the apparently soluble kinase (200 μM stock solution in AA buffer containing 20% glycerol) was diluted 40-fold with the same buffer containing neither glycerol nor KCl and the turbidity measured as a function of melittin concentration at the indicated concentrations of NaCl. In (B) 40 μM melittin was added to the kinase diluted as in (A) and, after clarification by low speed centrifugation, increased concentrations of NaCl were added. The supernatants and the pellets obtained after subsequent high speed centrifugation were analyzed by SDS-PAGE.

of MLCKase with this fragment, or with telokin. In contrast to the sigmoid aggregation curves obtained for the fragment alone, the mixtures exhibited a characteristic biphasic response with pronounced clearing of the protein suspension at high melittin concentrations as observed for the kinase alone (Figures 10C and 3). This indicated that upon saturation with melittin the kinase interacted with the 24 kDa fragment, or with telokin, resulting in their solubilization of the related aggregates. The interaction was independently confirmed by cosedimentation of telokin with the oligomeric kinase (data not shown). The specificity of this interaction was obvious, because under the same condition, no solubilization was observed for the $ReLC_{20}$ aggregates (Figure 10C).

Formation of MLCKase Oligomers in the Absence of Melittin

The equilibrium between the different oligomeric forms of the kinase were also investigated in the absence of melittin. As shown in Figure 7A (''0'' melittin concentrations), the kinase alone also formed insoluble oligomers provided that the ionic strength was not too high. This aggregated kinase became progressively more soluble upon increasing the salt concentration (Figure 7A). Significantly and as expected for an oligomeric type of interaction, the relationship between turbidity (i.e., solubility) and the kinase concentration was biphasic (Figure 11A). This was confirmed in sedimentation experiments. At relatively low concentrations, practically all the kinase remained in the supernatant (Figure 11B, C) because it was present in monomeric and dimeric forms.[8] At the high concentrations of the kinase, however, soluble oligomers were formed, as indicated by their sedimentation at high speed centrifugation (Figure 11C).

Effects of Melittin on MLCKase Catalytic and Autocatalytic Activity

The effects of melittin on the MLCKase activity and its binding to myosin filaments were also inves-

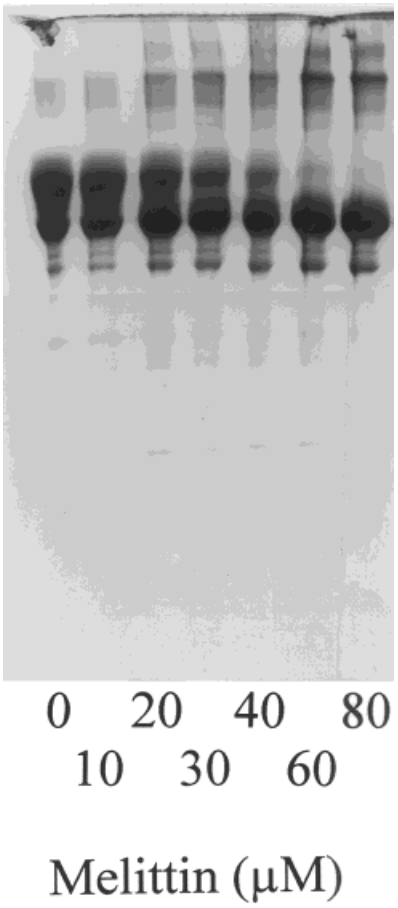

FIGURE 8 Melittin induced oligomerization of MLCKase as revealed by zero-length cross-linkage and SDS PAGE. Cross-linking was carried out for 30 min at the indicated melittin concentrations. Note that melittin increased the relative concentration of the kinase octamers and decreased that of the dimers.

tigated. Despite its high affinity for calmodulin, melittin produced only a moderate inhibition of MLCKase catalytic activity (Figure 12A). Similarly, there was no inhibition of the CM-independent kinase activity of the purified 61 kDa kinase fragment (Figure 12A) which can not dimerize.[8] The low extent of melittin inhibition, however, most likely resulted from the sequestrating of melittin by the $ReLC_{20}$ (Sobieszek, unpublished results; see also Figure 3).

As shown in Figure 12B, melittin strongly inhibited rates of Ca^{2+}/CM-dependent[24] and CM-independent[24,28] autophosphorylation of MLCKase. This inhibition, however, was restricted to melittin concentrations at which the insoluble aggregates were observed. At the higher concentrations, when soluble oligomers were formed, there was a 2-fold increase of these rates in comparison to their minimal values. Thus, the effects were again biphasic indicating that, besides the inhibition, there was a slower, melittin-stimulated autophosphorylation at an additional site. If so, then this slow autophosphorylation should, in principle, produce an increase in the total CM-independent ^{32}P-incorporation levels at relatively low melittin concentrations. Autophosphorylation progress curves obtained at different melittin concentrations (Figure 12C) showed that this was indeed the case.

Effect of Melittin on Binding of MLCKase to Myosin

From the foregoing, we concluded that oligomerization of the kinase may play a role in its tight association with myosin filaments. Therefore, the effects of melittin on binding of MLCKase to myosin filaments, which could be studied only for soluble oligomers, were also investigated. As shown in Figure 13, addition of 50 μM melittin to MLCKase/myosin mixtures significantly increased the amount of kinase bound to myosin. At 5 μM concentration most of the kinase was bound to 8 μM of myosin. This corresponded to a 10-fold higher stoichiometry than observed for the endogenous kinase copurified with myosin filaments.[29]

DISCUSSION

Attempts to elucidate the role of calmodulin in the regulation of cellular processes and, in particular, that of CM-dependent enzymes, have led to a widespread use of CM antagonists (for a review, see Ref. 9). Among them several toxic peptides from *Hymenoptera* species proved very potent in CM binding. One of these is melittin, a 26-residue polypeptide from honey bee venom, which forms a very tight complex with CM with a reported dissociation constant in the 0.1–3 nM range.[10,11] It has become clear, however, that these antagonists have other significant biological actions[9] that result from their direct interaction with the enzymes themselves or with their substrates. In agreement, the present report demonstrates that melittin also interacted with the smooth muscle MLCKase apoenzyme, the kinase-related protein (telokin), and the myosin light chains, significantly influencing their solubility. The latter observation is consistent with the data of Malencik and Anderson[26] demonstrating that the $ReLC_{20}$ interacted with melittin with $K_d = 1–2$ μM. At the same time melittin had no effect on the solu-

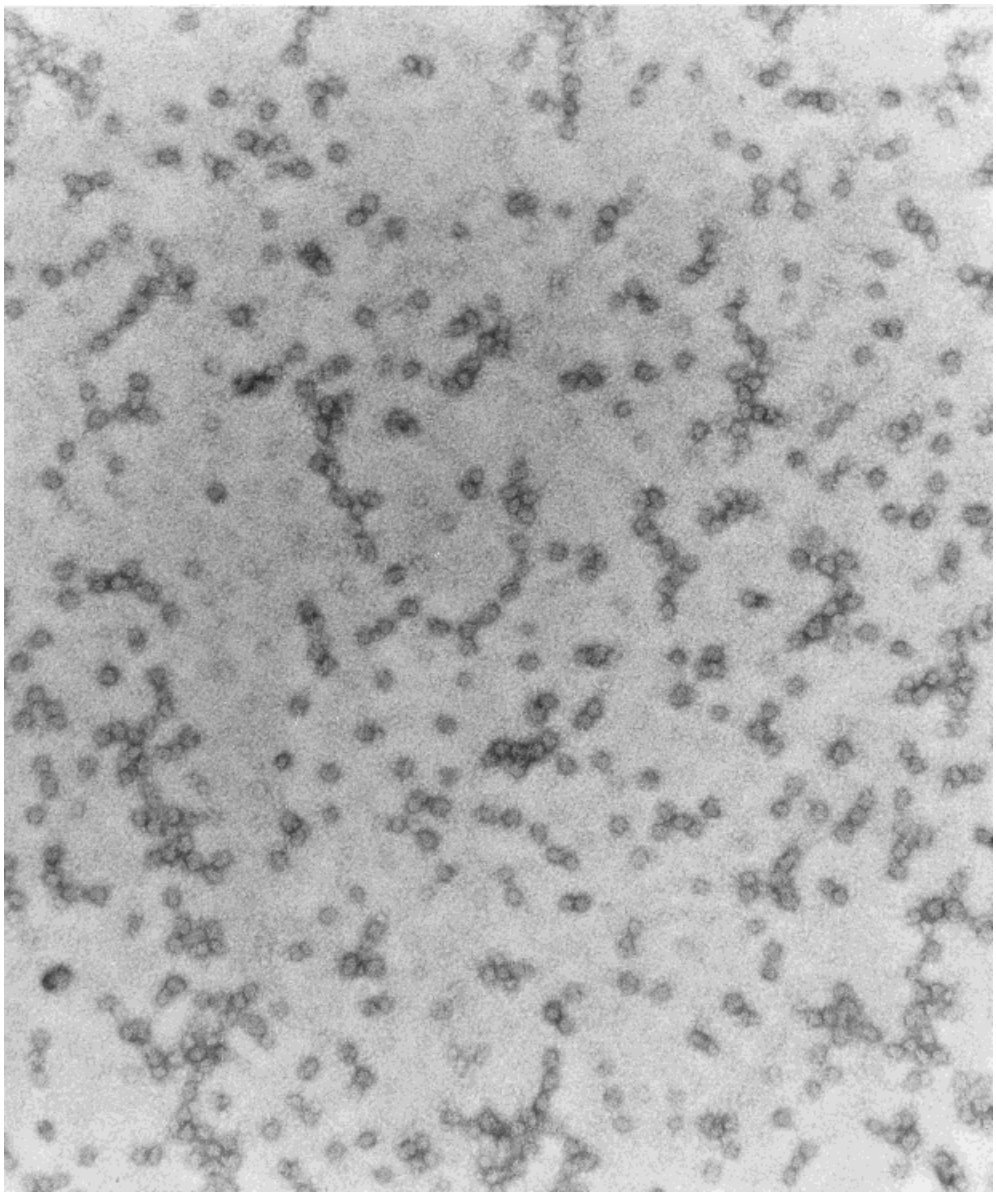

FIGURE 9 A representative electron micrograph of MLCKase soluble oligomers formed in the presence of the high concentrations of melittin. Note that the relatively constant size of the oligomers and the heavy background resulting from the presence of the kinase monomers. Magnification ×47,500.

bility of acidic proteins such as bovine serum albumin and caldesmon or of CM, which binds with exceptionally high affinity to melittin.[10,11] Caldesmon is, like MLCKase, a CM-binding protein[30]; therefore, the effect of melittin on the solubility of proteins does not simply result from an interaction between basic (melittin) and acidic proteins but characterizes a class of proteins, suggestive of a more specific interaction. In our view, this should have some analogy to the action of many newly discovered basic proteins or gangiosides that were shown to modulate the activity of many CM-dependent enzymes.[31,32]

There have been numerous studies on effect of CM antagonists on CM-dependent enzymes aimed at better understanding the interaction of CM with its target proteins and also in order to design more effective and specific CM inhibitors that are frequently used as therapeutic agents.[9] Taking MLCKase phosphorylation system and the most po-

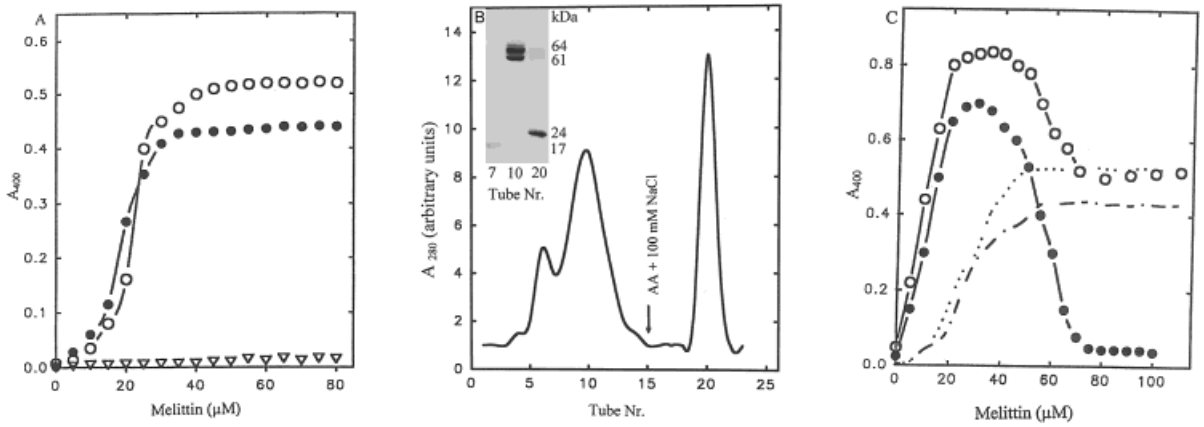

FIGURE 10 Identification of MLCKase domain responsible for formation of the insoluble oligomers. In (A) turbidities of the kinase hydrolyzed by trypsin (○ - ○), as well as of the purified 24 kDa (● - ●) and 64 kDa (▽ - ▽) kinase fragments, were measured as a function of added melittin. The concentration of kinase and fragments was 5 μM. Note that melittin induced aggregation of trypsinolyzed kinase and the 24 kDa fragment but not of the 64 kDa fragment. (B) shows that only the 24 kDa fragment bound to a melittin-affinity column. In (C), increased concentrations of melittin were added to MLCKase–telokin (● - ●) or MLCKase/$ReLC_{20}$ (○ - ○) mixtures. All proteins were at 5 μM concentration. Dotted and dashed/dotted curves represent respectively the aggregation of $ReLC_{20}$ and telokin alone. Note full clearance of the MLCKase–telokin suspension at high concentrations of melittin. In contrast, the turbidity of the MLCKase–$ReLC_{20}$ mixture was the same as the $ReLC_{20}$ control.

tent antagonist (melittin) known we have shown that this approach may be compromised as a result of interactions of the antagonist with the CM-dependent enzyme itself or its substrate.

Intrasteric Inhibition Model

Jarrett and Madhavan[33] proposed a general flip-flop model for the regulation of CM-dependent enzymes based on data on their interaction with melittin. In short, the main prediction of this model is that these types of enzymes possess, beside a CM-binding site, a CM-like site; the latter binding melittin. According to this model the intrasteric inhibition takes place in the absence of Ca^{2+}/CM due to interaction between these two sites. For the more widely accepted pseudosubstrate model,[4,5] on the other hand, the regulatory domain contains the sequence of the phosphorylation site of the protein substrates (see Introduction). The main difference between the two models amounts to the difference in the autoinhibitory sequence: CM-like in the first model and substrate-like in the second. The present data and the data of Malencik and Anderson[26] demonstrating the binding of melittin not only to calmodulin but also to the kinase substrate ($ReLC_{20}$) indicates that there is, possibly, no difference between the two models at least for MLCKase. Actually, Jarrett and Madhavan did not exclude such a possibility by pointing out that the CM-like site could be located in the pseudosubstrate autoinhibitory region of the kinase. Our results, however, indicate that this site was located next to this region, namely within the 24 kDa telokin-like fragment of the kinase.

The effect of melittin on the MLCKase oligomerization described here does not agree with the suggestion of Jarrett and Madhavan[33] concerning phosphofructokinase. This enzyme, like MLCKase,[8] forms dimers and tetramers and CM affects its oligomerization.[34] Jarrett and Madhavan[33] suggested that phosphofructokinase may represent a special case of an oligomeric CM-binding enzyme in which the CM-binding site of one protomer binds to the CM-like site of another protomer to form oligomers. However, from the present data on interaction of melittin with MLCKase it is clear that this type of interaction is far more complex. The kinase exhibited at least 6 high affinity melittin binding sites and their saturation resulted in an enhancement of the oligomer formation and not in a reduction as would be predicted by the model.

MLCKase Oligomeric Equilibria

It has been demonstrated earlier that, in solution, dimeric kinase exists in an equilibrium with its mo-

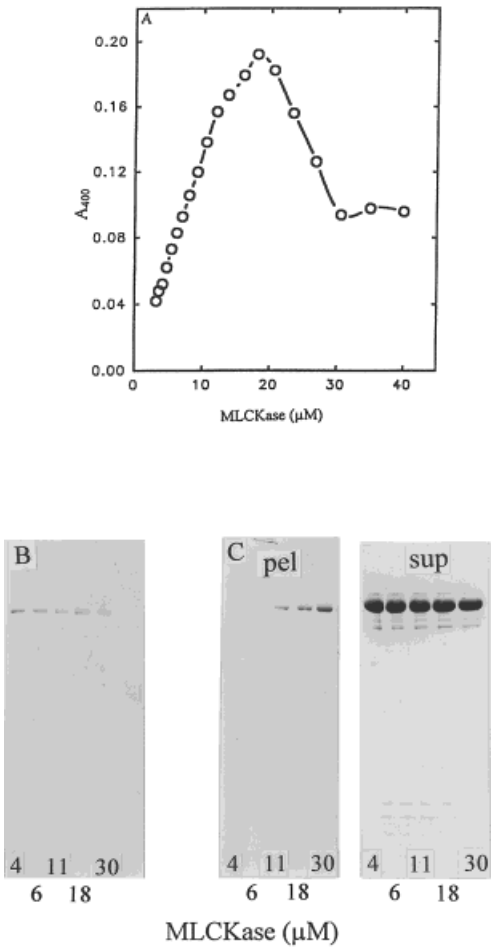

FIGURE 11 Solubility of MLCKase as a function of its concentration. The kinase stock solution was diluted (as in Figure 7) with AA buffer to the required concentrations and its turbidity (A) as well as sedimentation properties (B) and (C) analyzed. Glycerol was present at the highest concentration tested, and therefore it was added (up to 4%) to all dilutions. (B) shows SDS-PAGE of the pellets obtained after low speed centrifugation. In (C) the clear supernatants obtained after low speed centrifugation were subsequently subjected to a high speed centrifugation and the corresponding pellets and supernatants analyzed by SDS-PAGE. Loading volumes of the samples were such as to compensate the differences in the initial kinase concentrations. Note the biphasic solubility of the kinase with a maximum at 18 μM (A). The increase in the solubility at high concentrations of the kinase was due to formation of soluble oligomers, which could be pelleted only at high speed centrifugation (C).

nomeric and oligomeric forms and that this equilibrium can be shifted into direction of the dimers after adding of Ca^{2+} and CM.[8] The relative concentrations of monomers (43%), dimers (55%), and oligomers (2%) were estimated from light scattering measurements under physiological salt concentrations.[35] The related dimer–monomer equilibrium is shown in our model (Figure 14; top). We suggest that these dimers are asymmetrical and are formed via binding of the telokin-like domain of one MLCKase molecule to the catalytic domain of another molecule in analogy to the intrasteric blocking hypothesis for the individual molecules.[4,5,33] Other types of dimers are also formed, although at relatively low levels, and they include symmetrical and asymmetrical dimers formed by aggregation of the telokin-like domains of the kinase as deduced in the present study. Addition of melittin cannot solely be responsible for the formation of the latter two types of dimers because properties of MLCKase in the absence of melittin (at low salt concentrations) are similar to those in its presence (under physiological salt conditions). MLCKase forms insoluble aggregates and soluble oligomers under both conditions.

The mechanism of MLCKase oligomerization is not clear at present but, as illustrated in our model (Figure 14), it should also involve interaction between the telokin-like domain of one MLCKase molecule with a different region of another molecule and include at least some dimeric forms described above. Solubilization of telokin in the presence of MLCKase (at melittin concentrations normally resulting in its precipitation) and their binding (data not shown) indicates that, indeed, this type of interaction is taking place. Independently, telokin was shown to bind to MLCKase oligomers resulting in their monomerization.[17] We suggest that the soluble helical oligomers are formed from the asymmetrical dimers via the same type of interactions that, in the case of symmetrical dimers, result in segment- or ring-like structures seen in electron microscope. The insoluble aggregates were formed from both types of dimers by nonspecific aggregation.

Our model for MLCKase oligomerization is independently supported by observations demonstrating that MLCKase is bound to both anion and cation exchange columns.[36] Thus, the kinase possesses two oppositely charged domains that must play a role in the dimer formation. It is clear that at a melittin concentration for which the insoluble aggregates were formed most of the kinase was in the dimer or in the monomer form. We therefore suggest that a nonspecific aggregation of the dimers, possessing lower net charge than the monomers, results in their aggregation and precipitation. At higher melittin concentration, the relative concentration of the dimers decreased as judged from our zero-length crosslinking experiments. It is not clear, however, whether this is due to an increase of the

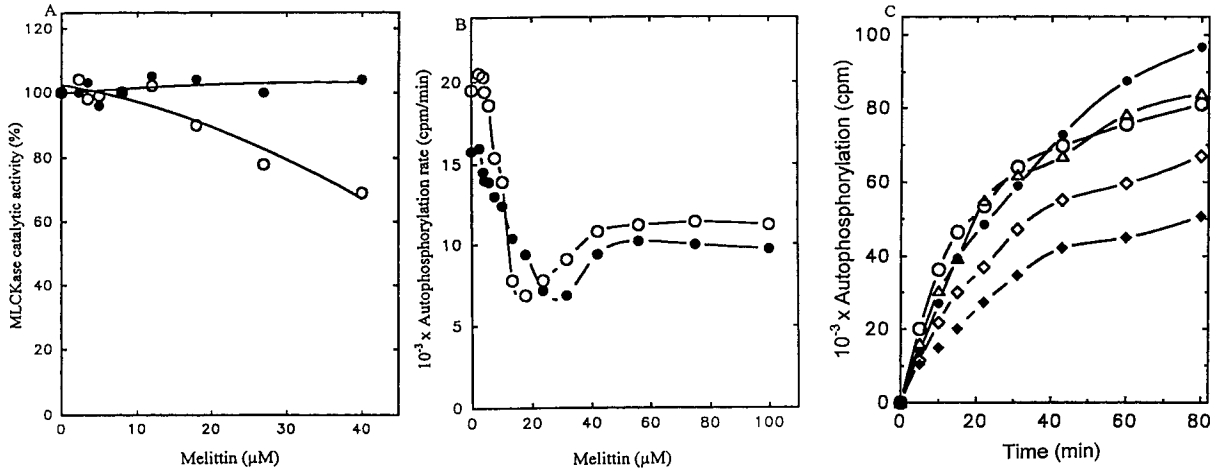

FIGURE 12 Effect of melittin on catalytic (A) and autocatalytic (B and C) activities of MLCKase. In (A) catalytic activities of CM-dependent MLCKase (○ - ○) and its CM-independent 61 kDa fragment (● - ●) were measured as a function of melittin added. In (B) MLCKase intermolecular (Ca^{2+}/CM present; ○ - ○) and intramolecular (2 m*M* EGTA; ● - ●) autophosphorylation rates were measured as a function of melittin concentrations. (C) shows MLCKase (10 μM) autophosphorylation progress curves obtained at different melittin concentrations (in μM): control (○ - ○), 2 (● - ●), 4 (△ - △), 8 (◇ - ◇), and 16 (◆ - ◆). For more details, see text.

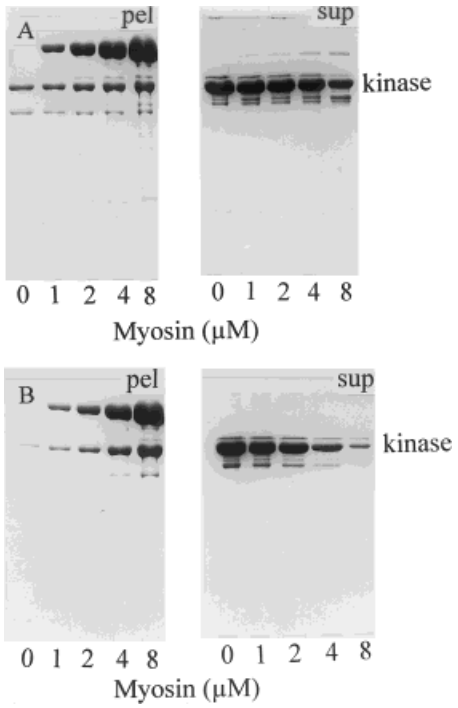

FIGURE 13 Binding of the kinase to myosin filaments in the absence (A) and presence (B) of melittin. Note the increase in binding resulting from melittin-induced oligomerization of the kinase. The uppermost band seen in the pellets corresponds to myosin heavy chain. Concentrations of added myosin are indicated and that of melittin (B) was 50 μM.

long-range order of the aggregates, as suggested in our model, or due to simple dissociation of the dimers. In both cases there would be a shift of the equilibrium into direction of oligomers.

The biphasic solubility of the melittin–MLCKase interaction shows some analogy to the antibody–antigen precipitation reaction that reaches an optimum precipitation with 2–4 molar excess of antibody. The complexes formed then become more soluble when an excess of antigen is added. In this case, however, the insoluble complexes were shown to contain mainly relatively short antibody–antigen oligomers and the soluble ones correspond to the Ag_2Ab trimers only. In addition, the 200-fold difference in molecular mass of the antibody molecule as compared to melittin suggests a different mechanism at play.

The melittin-induced MLCKase oligomerization cannot be claimed to be physiologically relevant except of during the bee venom poisoning. From these and other our studies[8,35] it is clear, however, that at physiological concentrations (i.e., 5 μM; see Ref. 1) the kinase does form similar, if not identical, insoluble and soluble oligomers even in the absence of melittin. Such oligomers could, therefore, contribute to the modulation of the MLCKase activity and its association with myosin filaments.

This work was done during the tenure of Lise Meitner Fellowship from the Austrian Science Foundation to EBB

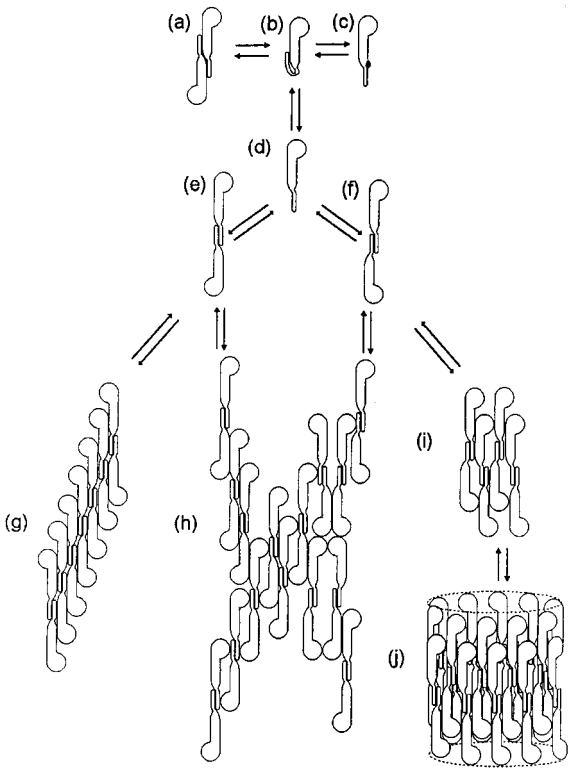

FIGURE 14 MLCKase oligomeric equilibria deduced on basis of its interaction with melittin. Inactive (blocked) kinase monomers (b) become unblocked (but remain inactive) (d) in a configuration similar to that induced by CM binding (c). This configuration is facilitated by melittin and leads to aggregation of MLCKase telokin-like fragments. As result, two types of dimers are formed: asymmetrical (e) and symmetrical (f). At low melittin concentrations, these two types of dimers aggregate nonspecifically forming insoluble precipitates (h). At higher melittin levels, the dimers assemble into more ordered soluble helical-like structures of variable length (g), or to segment-like structures (i). The latter can form more stable ring-like structures of certain length (j) observed by electron microscopy. Under normal physiological conditions more than half of the kinase is in the form of blocked monomers (b) and the other half is in the form of inactive dimers (a). We suggest that the soluble oligomers (also present under these conditions) and the insoluble kinase aggregates (observed at low ionic strength) are formed in a manner similar to that shown in this model. For further details, see text.

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