Structural Changes in Melittin and Calmodulin upon Complex Formation and Their Modulation by Calcium†

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ABSTRACT: In the presence of Ca^{2+}, calmodulin forms a 1:1 high-affinity complex (K_d = 3 nM) with melittin, a peptide from bee venom; in the presence of ethylenediaminetetraacetic acid, a second type of complex, of much lower affinity, is formed [Comte, M., Maulet, Y., & Cox, J. A. (1983) *Biochem. J. 209*, 269–272]. In this paper, these interactions were studied by tryptophan fluorescence and circular dichroism spectroscopy in near- and far-UV. Interaction between the two peptides in the presence as well as in the absence of Ca^{2+} leads to the shielding of the tryptophan residue of melittin from its aqueous environment and to an increase in the α-helical content of bound melittin; for instance the Ca^{2+}-dependent high-affinity complex formation enhances the α-helical content of melittin from 5 to 72%. Provided Ca^{2+} is present, the interaction between the two peptides leads to significant changes in the environment of at least one tyrosine residue of calmodulin as measured by near-UV circular dichroism. In the absence of Ca^{2+}, calmodulin binds two melittin molecules with a K_d of ca. 10 μM; at higher concentrations of free melittin, additional binding occurs (up to 5 mol of melittin/mol of calmodulin), with concomitant denaturation of calmodulin. In the presence of 4.0 M urea, the low-affinity complexes formed in the absence of Ca^{2+} dissociate, due to the denaturation of metal-free calmodulin, whereas the spectroscopic signals of the high-affinity Ca^{2+}-dependent complex are not affected. Equilibrium dialysis on an equimolar mixture of melittin and calmodulin showed that melittin enhances the affinity of calmodulin for Ca^{2+}, resulting in the following macroscopic dissociation constants: K_1 = 0.2 μM; K_2 = 0.72 μM; K_3 = 0.18 μM; K_4 = 1.1 μM. The energy coupling involved in the formation of the ternary complex amounts to at least 7.9 kcal/mol, indicating that the saturation of calmodulin by Ca^{2+} increases its affinity for melittin at least (1.5 × 10^6)-fold. We suggest that in the presence of Ca^{2+}, melittin binds to that part of the surface of calmodulin that also interacts with the target enzyme.

Calmodulin (CaM),[1] the ubiquitous intracellular modulator, regulates in a calcium-dependent way a great number of enzymes and cell functions (Klee et al., 1980; Cheung, 1980). For the activation of phosphodiesterase, the following mode of action has been proposed. This can also be applied to other enzymes (Kakiuchi et al., 1973):

$$n\mathrm{Ca}^{2+} + \mathrm{CaM} \rightleftharpoons \mathrm{CaM}^*\cdot\mathrm{Ca}^{2+}_n$$

$$\mathrm{CaM}^*\cdot\mathrm{Ca}^{2+}_n + \mathrm{E} \rightleftharpoons \mathrm{CaM}^*\cdot\mathrm{Ca}^{2+}_n\cdot\mathrm{E}$$

The scheme predicts that, upon binding of a certain number of Ca^{2+} ($1 \leq n \leq 4$), CaM undergoes a critical conformation change. The activated CaM* binds, then, to a target enzyme and modifies its activity. Recently, on the basis of quantitative studies, more refined models were proposed for the activation of phosphodiesterase (Wang et al., 1980, Huang et al., 1981; Cox et al., 1981), myosin light chain kinase (Blumenthal & Stull, 1982), Ca^{2+},Mg^{2+}-ATPase (Cox et al., 1982), and adenylate cyclase (Malnoë et al., 1982). Cox and co-workers proposed that the occupation of three Ca^{2+}-binding sites is necessary and sufficient to induce the critical conformation change, which allows the activation of these enzymes. Their analysis, however, gave no information about the affinity changes for Ca^{2+} as CaM* is bound to its target. During the course of its saturation with Ca^{2+}, CaM displays more than two conformational states (Klee, 1977; Seamon, 1980), and a hydrophobic domain appears on its surface. On the basis of these reports and the determination of the intrinsic binding constants for Ca^{2+}, Haiech et al. (1981) proposed a model of ordered binding of Ca^{2+}, which in turn would allow specific activation of different cellular functions. This latter postulate has not received any experimental support yet, since the nature of the conformational changes of CaM and the topology of its interaction with a target is not known. Investigations were impaired mostly by the lack of sufficient amounts of purified CaM-stimulated enzymes to allow structural studies at the molecular level. Another approach consists in the study of other proteins or peptides sharing common CaM-binding properties with the enzymes but whose affinity for CaM is physiologically not relevant, such as troponin I (Keller et al., 1982; Olwin et al., 1982). Although the affinity of the latter protein for CaM (K_d = 20 nM) is comparable to that of a target enzyme, it presents the disadvantage of being a relatively big molecule (M_r 23 000), whose tertiary structure is not known. Some investigators studied also the binding to CaM of myelin basic protein, histone H2B (Grand & Perry, 1980), and of small peptides whose sequences are known (Weiss et al., 1980; Malencik & Anderson, 1982). However, they are not adequate models of the interaction between an enzyme and CaM, since their affinities are 3 orders of magnitude too low. The vasoactive intestinal peptide, the gastric inhibitory peptide, and secretin, which bound calmodulin with the highest affinity, have K_d values of 50, 70, and 100 nM, respectively. Their affinities for CaM are dependent on the presence of Ca^{2+}, and they exhibit a one-to-one stoichiometry (Malencik & Anderson, 1983). However, their secondary structure, when interacting with CaM, has not been elucidated yet. By cross-linking studies, it was shown that β-endorphin also interacts with CaM in Ca^{2+}-dependent fashion (Giedroc et al., 1983). This binding is less easily studied since more than one endorphin molecule can bind on one CaM.

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[1] Abbreviations: CaM, calmodulin; ME, melittin; $NaDodSO_4$, sodium dodecyl sulfate; CTMA, cetyltrimethylammonium bromide; DMDAO, dimethyldodecylamine oxide; EDTA, ethylenediaminetetraacetic acid; HEDTA, *N*-(2-hydroxyethyl)ethylenedinitrilo-*N*,*N*′,*N*′-triacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid.

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Recently, we reported that melittin (ME), the amphipathic peptide from bee venom, interacts with CaM in a Ca^{2+}-dependent way. The stoichiometry is 1:1 in the presence of Ca^{2+}, and the dissociation constant is in the nanomolar range (Comte et al., 1983). A complex of lower affinity was also observed in the absence of Ca^{2+}. The low molecular weight of ME (M_r 2840), its elucidated α-helical structure induced by interactions with hydrophobic surfaces (Terwilliger & Eisenberg, 1982), and its high affinity for CaM make it a good candidate for detailed structural studies. This work describes the interaction of ME with CaM in the presence and in the absence of Ca^{2+}, at the structural level, and presents phenomenological evidences that ME binds to CaM in the same way as troponin I does.

Experimental Procedures

Materials. Bovine brain calmodulin was purified according to Watterson et al. (1976), and its concentration was assessed by UV spectrophotometry in the absence of Ca^{2+} (ϵ_{278} = 3000 M^{-1} cm^{-1}). Melittin was purified from bee venom (Sigma, grade I) as previously described (Maulet et al., 1982), and its concentration was spectrophotometrically assessed (ϵ_{280} = 5470 M^{-1} cm^{-1}). α-*N*-[^{3}H]Acetylmelittin was prepared according to a published method (Maulet et al., 1980). Spectrapore dialysis bags are products of Spectrum Medical Industries Inc., Los Angeles, CA.

Fluorescence Measurements. Emission fluorescence spectra were recorded at 25 °C on a Baird Atomic fluorescence spectrophotometer, with entrance and exit slits of 2.0 and 1.0 nm, respectively. Quartz cells (1 × 1 cm) were used. Excitation was at 290 nm in order to minimize the excitation of tyrosines. The proteins were diluted to 10 μM final concentration in 20 mM Tes buffer, pH 7.0–100 mM NaCl, containing 1.0 mM either $CaCl_2$ or EDTA. Urea was added to a final concentration of 4.0 M when indicated. Recordings were made after incubation for at least 15 min at 25 °C.

Near-Ultraviolet Circular Dichroism. Spectra were recorded at room temperature on a Jasco J-20 spectropolarimeter with a 1.0-nm slit as described previously (Cox & Stein, 1981). Quartz cuvettes of 1.0-cm light path were used. The concentrations of calmodulin and melittin were 275 μM in 60 mM Tes, pH 7.0, 150 mM NaCl, and 0.5 mM $CaCl_2$, with 4.0 M urea when stated. In the absence of Ca^{2+}, the spectra were recorded at a concentration of 1.0 mM EGTA. The data were expressed in differential molar absorbance, $\Delta\epsilon$ (M^{-1} cm^{-1}), where concentrations are in molarities of calmodulin.

Far-Ultraviolet Circular Dichroism. Spectra were recorded at room temperature between 250 and 195 nm, with a slit width of 1.0 nm. A 0.2-cm light-path quartz cuvette was used in all measurements. The buffer was 20 mM Tes, pH 7.0–100 mM NaCl containing 1.0 mM either $CaCl_2$ or EDTA. Both peptides were diluted to 10^{-5} M. When detergents were added, their final concentrations were 0.4, 0.04, and 0.02% for NaDodSO$_4$, DMDAO, and CTMA, respectively. These values are well above their respective critical micellar concentrations, as demonstrated by binding of the fluorescence dye 6-(4-toluidino)-2-naphthalenesulfonic acid to the micelles (not shown). In titration experiments, calmodulin was kept at 10^{-5} M, and melittin was varied from 0 to 2 × 10^{-4} M. The reversibility of the interactions upon Ca^{2+} addition or removal was assessed as follows: spectra were recorded in the presence of 1.0 mM of either $CaCl_2$ or EDTA. Then EDTA or $CaCl_2$, respectively, was added to a final concentration of 2.0 mM, and samples were measured again after a fixed incubation time. Data were either expressed in observed ellipticities, θ_λ (millidegrees), or in molar residual ellipticities, $[\theta]_\lambda$ (degrees centimeters squared per decimole). The approximative α-helix content of the peptides (f_h) was evaluated according to Chen & Yang (1971) with the following equation:

$$f_h = -([\theta]_{222} + 2340)/30300$$

Melittin-Binding Measurements. Binding of melittin to calmodulin in the absence of Ca^{2+} was determined by equilibrium dialysis in a microcell device of 250-μL half-cell volume, mounted with Spectrapor 2 membranes (M_r cutoff 12 000–14 000). A control experiment showed that melittin equilibrated freely across the membrane and that less than 4% calmodulin leaked out during the time of equilibration. Dialysis was started with each side of the chamber containing the same concentration of α-*N*-[^{3}H]acetylmelittin (25.4 mCi/mmol) in 150 μL of 20 mM Tes, pH 7.0, 100 mM NaCl, and 1.0 mM EDTA. One compartment contained 10^{-5} M calmodulin. Equilibration was allowed to proceed for 4 days at 7 °C. A total of 100 μL was removed from each compartment and counted in 2.0 mL of scintillation cocktail.

Calcium-Binding Measurements. The binding of Ca^{2+} to a calmodulin–melittin 1:1 mixture was determined by equilibrium dialysis. The buffer was 20 mM Tes, pH 7.0, 100 mM NaCl, and 1.0 mM HEDTA, the chelator being added in order to enhance the sensitivity in Ca^{2+} detection. Varying amounts of $CaCl_2$ were added in order to meet the required free Ca^{2+} concentration. Samples (0.5 mL) of the peptides (both 60 μM) in the same buffer were put in Spectrapor 6 (M_r cut off 1000) dialysis bags and allowed to equilibrate against 100 mL of buffer at 7 °C. After 48 h with two changes of buffer, inside and outside Ca^{2+} ion concentrations were measured by atomic absorption with a Perkin-Elmer 303 spectrometer. Bound calcium was taken as the difference between inside and outside concentrations. Free calcium was computed from the outside concentration of calcium and corrected for the amount complexed to HEDTA with the computer program COMICS (Perrin & Sayce, 1967). Inside protein concentrations were determined by the method of Bradford (1976), with a melittin–calmodulin 1:1 mixture as standard. Dialysis bags were checked for retention of melittin, under the experimental conditions. Less than 8% of the material leaked from a bag containing 60 μM melittin alone, as determined by the method of Bradford. The binding was analyzed by means of the Adair equation (Adair, 1925) with an interative least-squares method for the evaluation of the macroscopic dissociation constants. Errors on each constant remained below 30%.

Results

Fluorescence of Aromatic Amino Acid Residues. At an excitation wavelength of 290 nm, the fluorescence of the tyrosines of CaM exhibited a broad maximum at 305 nm in the presence of 1.0 mM Ca^{2+} (Figure 1A,C). In the presence of EDTA, the quantum yield was decreased (Figure 1B,D). These signals were negligible as compared to the fluorescence spectra of the same molar amounts of ME. The maximum of fluorescence at 350 nm indicated that ME in solution exposes its unique tryptophan to the aqueous environment. At 10 μM of both peptides, we observed a blue-shift in the maximum of fluorescence to 340 nm, both in the presence and absence of Ca^{2+} (Figure 1A,B). Since the sum of the spectra of ME and CaM alone exhibited the same maximum as ME alone (350 nm), we interpreted this shift as being due to the shielding of the tryptophan of ME from the aqueous environment (Dufourcq & Faucon, 1977).

In the presence of 4.0 M urea, the spectra of the ME–CaM 1:1 mixture showed the same shift of the maximum of fluorescence to a lower wavelength (242 nm) provided 1.0 mM Ca^{2+} was present (Figure 1C). In 1.0 mM EDTA, however,

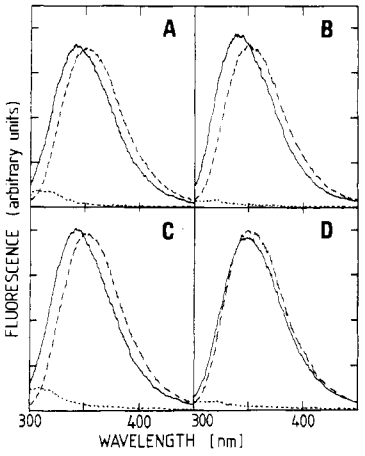

FIGURE 1: Emission fluorescence spectra of ME and CaM upon complex formation. The measurements were performed in 20 mM Tes, pH 7.0–100 mM NaCl at 25 °C: (--) 10 μM ME; (···) 10 μM CaM; (—) 10 μM ME and 10 μM CaM. Measurements were performed in (A) 1.0 mM $CaCl_2$, (B) 1.0 mM EDTA, (C) 1.0 mM $CaCl_2$ and 4.0 M urea, and (D) 1.0 mM EDTA and 4.0 M urea.

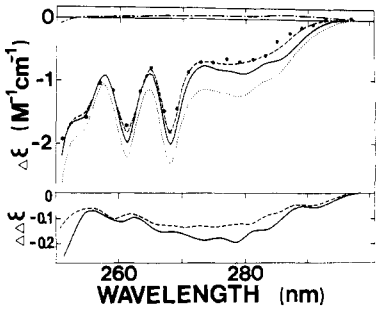

FIGURE 2: Near-ultraviolet circular dichroism of ME and CaM and effect of complex formation. Spectra were recorded at room temperature in 60 mM Tes, pH 7.0–150 mM NaCl: (top) (—) 275 μM CaM in 0.5 mM $CaCl_2$, (--) 275 μM CaM in 1 mM EGTA, (--) 275 μM ME in 0.5 mM $CaCl_2$, (···) 275 μM ME and 275 μM CaM in 0.5 mM $CaCl_2$, and (●) values of the difference between the spectrum of 515 μM ME and 275 μM CaM in 1 mM EGTA and that of ME 515 μM; (bottom) difference spectrum of 515 μM ME and 2 75 μM CaM minus 275 μM CaM (—) in 0.5 mM $CaCl_2$ and (--) in 0.5 mM $CaCl_2$ and 4.0 M urea.

the maximum of the spectra of the mixture remained at 350 nm for the experimental value and that one that was computed by summation of the spectra of the individual peptides (Figure 1D). These measurements confirmed previously reported data (Comte et al., 1983) indicating that the binding between CaM and ME is strongly reduced by the simultaneous presence of urea and EDTA.

Near-Ultraviolet Circular Dichroism of Aromatic Residues. Figure 2 shows the circular dichroic spectra of CaM and ME. ME alone did not display any noticeable chirality above 290 nm nor any distinct peak in the 280–290-nm region. The presence of Me slightly enhanced the broad negative peaks of Ca^{2+}-saturated CaM around 280 nm, which are generally attributed to its tyrosine residues. Contributions of the tryptophan of ME were ruled out, since its signals commonly exhibit discrete sharp peaks at higher wavelengths [for a review on the subject, see Strickland (1974)]. The difference spectrum of the CaM–ME 1:1 mixture minus CaM (Figure 2, bottom) shows that although ME enhanced the chirality of the complex over the whole 250–290-nm range, it did not significantly affect the two sharp negative peaks at 262.5 and 269 nm, which correspond to the phenylalanine residues. The presence of 4.0 M urea did not alter these spectral changes provided Ca^{2+} was present (Figure 2, bottom).

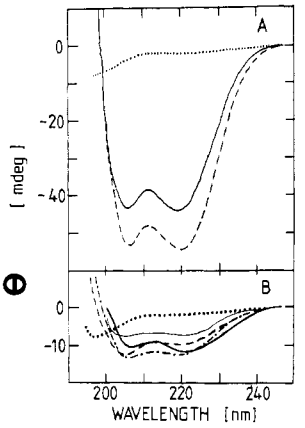

FIGURE 3: (A) Far-ultraviolet circular dichroism of ME and CaM obtained in the presence of an excess of Ca^{2+}. Buffer was 20 mM Tes, pH 7.0, 100 mM NaCl, and 1 mM $CaCl_2$; ellipticities are expressed in millidegrees. (—) 10 μM CaM; (···) 10 μM ME; (--) 10 μM CaM and 10 μM ME. (B) Far-ultraviolet circular dichroism of ME. (—) Difference spectrum of 10 μM CaM and 10 μM ME minus 10 μM CaM in the presence of 1.0 mM $CaCl_2$; (—) difference spectrum of 10 μM CaM and 10 μM ME minus 10 μM CaM in the presence of 1.0 mM EDTA in place of $CaCl_2$; (···) 10 μM ME and 0.02% CTMA; (--) 10 μM ME and 0.04% DMDAO; (-·-) 10 μM ME and 0.4% $NaDodSO_4$.

Since, at low concentration of free Ca^{2+}, CaM forms a complex of low affinity with ME (see below; Comte et al., 1983), we examined the dichroic spectra of aromatic residues in the presence of EGTA. The chirality intensities of a mixture of both peptides, corrected for the presence of ME, fitted with the spectrum of CaM alone (Figure 2). This means that the formation of the low-affinity complex in the absence of Ca^{2+} did not induce measurable structural changes in the environment of the tyrosines of CaM, in contrast with the high-affinity complex in the presence of Ca^{2+}.

Since urea dissociates the low-affinity complexes and seems not to affect the already random conformation of monomeric ME, its effect on the conformation of CaM alone was investigated. In the presence of 1.0 mM Ca^{2+}, the spatial organization of the tyrosines and the phenylalanines remained unaffected by 4.0 M urea. However, we observed a reduced dichroic signal in the simultaneous presence of urea and EGTA, indicating a denaturation of CaM (not shown). Accordingly, the dissociating effect of urea may be attributed to the denaturation of Ca^{2+}-free CaM.

Far-Ultraviolet Circular Dichroism. In the presence of 1.0 mM Ca^{2+}, CaM exhibited the far-UV circular dichroic spectrum shown in Figure 3A. Its molar residual ellipticity at 222 nm, $|\theta|_{222}$, was −14 716 deg cm^2 $dmol^{-1}$, a value close to previously reported data (Klee et al., 1980), indicative of an α-helix content (f_h) of 41%. In the same conditions, ME alone gave values of $|\theta|_{222}$ = −3846 deg cm^2 $dmol^{-1}$ and f_h = 5%. The equimolar mixture of the peptides yielded a spectrum more negative than the sum of each taken separately (Figure 3), indicating that the interaction enhanced the α-helical content of the complex. Since, in the presence of Ca^{2+}, this complex has a K_d in the nanomolar range and since the measurements were made at 10^{-5} M of both peptides, only the signal originating from the complex contributed to the circular dichroic spectrum. The difference spectrum between the complex and CaM alone was compared with the spectra of each peptide in conditions altering their conformations. In the presence of the anionic detergent $NaDodSO_4$ and the nonionic one DMDAO, the negative value of $|\theta|_{222}$ for ME increased to −20 192 and −18 269 deg cm^2 $dmol^{-1}$, respectively, indicating high helical contents. The spectra are comparable

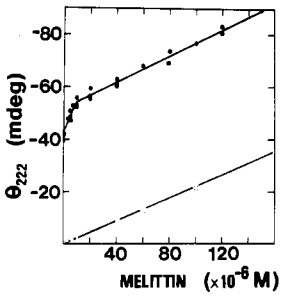

FIGURE 4: Titration of CaM by ME as observed by circular dichroism in the presence of Ca^{2+}. The buffer was 20 mM Tes, pH 7.0, 100 mM NaCl, and 1.0 mM $CaCl_2$. (Abscissa) Total ME concentration; (ordinate) negative observed ellipticity at 222 nm. (□) ME alone; (●) ME in the presence of 10 μM CaM. Curves are linear regressions of measurements done up to 0.2 mM ME in three independent experiments.

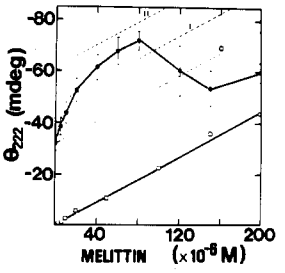

FIGURE 5: Titration of CaM by ME as observed by circular dichroism in the absence of Ca^{2+}. Conditions are the same as in Figure 4, except that $CaCl_2$ was replaced by 1.0 mM EDTA. (□) ME alone; (●) ME in the presence of 10 μM CaM. Error bars are the result of at least three independent measurements. The dashed lines are computed titration curves for the binding of zero (0), one (I), and two (II) ME's per CaM, assuming no conformational change in CaM, 100% α-helix in bound ME, and infinite affinities between the constituents.

to the difference spectrum of the ME–CaM mixture minus CaM (Figure 3B). No change in the conformation of ME occurred in the presence of the cationic detergent CTMA. The spectra of Ca^{2+}-saturated CaM were systematically reduced in intensities in the presence of these three detergents (not shown). Above their respective micellar concentrations, $NaDodSO_4$, DMDAO, and CTMA reduced the $|\theta|_{222}$ of CaM to 79, 87, and 92%, respectively, of its native value. Below their critical micellar concentrations, the detergents had smaller ($NaDodSO_4$) or no effect at all (DMDAO and CTMA) (not shown). These experiments indicated that the enhancement of the negative ellipticity upon complex formation was due to an induction of α-helix in ME and not in CaM.

In the presence of 1.0 mM EDTA, CaM had a $|\theta|_{222}$ of −12 880 deg cm^2 dmol^{-1}, comparable to previously reported values (Klee et al., 1980) and corresponding to an α-helix content of about 35%. The difference spectrum of a ME–CaM 1:1 mixture minus CaM alone, in the absence of Ca^{2+}, is qualitatively similar to that in the presence of Ca^{2+} (Figure 3B). The interaction of ME with detergents was the same as that in the presence of Ca^{2+}, and CaM exhibited reductions of $|\theta|_{222}$ to 63, 92, and 84% when $NaDodSO_4$, DMDAO, and CTMA are respectively present above their respective micellar concentrations (data not shown). Also, in the absence of Ca^{2+}, ME seems to be the only component of the complex that undergoes a conformational change upon binding. However, a smaller signal change than that in the presence of Ca^{2+} was observed. This latter fact could be due either to a smaller conformational change or to a lower affinity leading to an incomplete binding between the peptides.

Since ME is an amphipathic peptide with denaturating properties, its effect on CaM was studied in titration experiments over a wide range of ME to CaM ratios. In the presence or absence of Ca^{2+}, the ellipticity of ME at 222 nm increased linearly with concentration (Figures 4 and 5), indicating that, over the concentration range covered, ME remained in its random-coiled monomeric conformation (tetramerization would have led to an upward curvature of the line). The slope of the lines gave a mean $|\theta|_{222}$ for ME of −4297 ± 240 deg cm^2 dmol^{-1}, indicating about 6.5% α-helix. As increasing concentrations of ME were added to 10^{-5} M CaM in the presence of saturating Ca^{2+}, a biphasic plot was obtained (Figure 4). At low ME to CaM ratio, the ellipticity sharply increased until the equimolar concentration. The relation was linear, correlation coefficients being above 0.99 in three independent experiments and 0.94 when all the data were put together. The slope, when related to the concentration of ME residues, gave an ellipticity of −24000 ± 3300 deg cm^2 dmol^{-1}. Hence, if CaM does not change its conformation upon complex formation, bound ME would display 71% α-helix in its bound state. The second phase paralleled the curve of ME alone, with a slope of −4711 ± 436 deg cm^2 dmol^{-1}. The profile of the titration demonstrates that (i) a one-to-one complex is formed, (ii) the dissociation constant is well below the concentrations covered and (iii) no other complex, promoting conformational changes of either peptide, was formed at higher concentrations.

In the absence of Ca^{2+}, similar titrations looked more complex. After the samples were mixed, their ellipticities diminished slightly, and the concentrated samples remained turbid for several hours, indicating the presence of transient aggregates. Therefore, all samples containing EDTA were incubated overnight at room temperature. The samples were then clear, and the measurements were stable but remained nevertheless less reproducible from one experiment to another than those in the presence of Ca^{2+}.

Three phases of the titration can be recognized in Figure 5. ME enhanced the negative ellipticity of the complex until a ME to CaM ratio of 7. From the studies of the interactions of each peptide with detergents, it can be inferred that only ME contributes to the enhancement of the signal. Accordingly, we calculated the theoretical circular dichroic titration profiles (Figure 5, dashed lines) when zero (0), one (I), or two (II) ME's interact with CaM, assuming infinite affinities and 100% α-helix formation in ME ($|\theta|_{222}$ = −32 640 deg cm^2 dmol^{-1}). Comparison of the experimental curve with the theoretical titration profiles indicated that at least two ME interacted with CaM in a nondenaturating fashion. In a second phase, the signal fell below the (0) line, indicating that, upon binding more ME, CaM became denatured and conserved finally less than 7% α-helix. In the third phase, the curve tended to parallel the profile of ME alone. The addition of an excess of Ca^{2+} to the samples in EDTA restored the profile of Figure 4 within minutes and over the whole range of ME concentrations. Conversely, the addition of excess EDTA to the Ca^{2+}-saturated complex led in a very short time to the appearance of a triphasic curve, similar to the one in Figure 5, which stabilized after overnight incubation.

Equilibrium Dialysis of CaM against ME. Since in the absence of Ca^{2+} CaM binds at least two ME's without noticeable denaturation (Figure 5), it was of interest to evaluate the dissociation constant of this interaction. Therefore, equilibrium dialyses were performed with membranes with a cutoff value between the molecular weights of ME and CaM. In this experiment, a ^{3}H-labeled derivative of ME was used, which interacts in an identical fashion with CaM as monitored by circular dichroism (data not shown) and direct-binding experiments (Comte et al., 1983). From the results presented

Table I: Binding of α-*N*-[^{3}H]Acetylmelittin (Ligand) to CaM in the Absence of Ca^{2+}

free ligand (μM)	bound ligand/CaM[a]
4.84 ± 0.21	0.177 ± 0.006
48 ± 4	1.7 ± 0.3
204 ± 5	5.3 ± 1

[a] Results are the means and standard errors of triplicate experiments. [CaM] was 10 μM in all cases. Conditions are described under Experimental Procedures.

Table II: Apparent Macroscopic Dissociation Constants of CaM for Ca^{2+} in the Presence or Absence of Melittin (μM)

	CaM[a]	CaM + ME[b]
K_1	2.0	0.2
K_2	5.9	0.72
K_3	17.7	0.18
K_4	218.8	1.1
$\bar{K}_d$[c]	14.4	0.41

[a] These constants were computed from intrinsic binding constants previously determined (Cox et al., 1981). [b] These constants were obtained by iterative least-squares fitting to the Adair equation (Adair, 1925) of the data shown in Figure 6 for Ca^{2+} binding to CaM in the presence of an equimolar amount of ME (60 μM). [c] $\bar{K}_d = (K_1K_2K_3K_4)^{1/4}$.

in Table I, it can be estimated that the K_d for the binding of the first two ME's is in the 10 μM range. Although under these conditions no denaturation could be observed by circular dichroism, the binding of the first ME to CaM in the absence of Ca^{2+} can be topologically different from the 1:1 complex in the presence of Ca^{2+}. Table I shows that CaM binds up to five ME's at higher concentrations of the latter peptide, this binding being accompanied by denaturation (Figure 5).

Binding of Ca^{2+} to ME–CaM Complexes. The effect of ME on the binding of Ca^{2+} by CaM was studied by equilibrium dialysis of the cation through membranes impermeable to both peptides. Figure 6 shows that ME, at equimolar concentration with CaM, enhanced the affinity of the four sites for Ca^{2+}. Half-saturation was shifted from 1.5×10^{-5} to 4.3×10^{-7} M free Ca^{2+}. The data were fitted to the Adair equation (Adair, 1925) with an iterative least-squares method. In Table II, the values of the macroscopic dissociation constants are compared to those measured in the absence of ME, which were computed from previously published results (Cox et al., 1981). ME promoted positive cooperativity between the Ca^{2+} sites, with a Hill coefficient of 1.38 between 25 and 75% saturation. In our experimental setup the positive cooperativity was partially due to the fact that the total ME concentration (60 μM) was below the estimated dissociation constant of Ca^{2+}-free CaM for ME. Therefore the experimental K_i values in Table II must be considered as approximate dissociation constants, which are higher than the real values at saturating [ME]. The mean dissociation constant is $(K_1K_2K_3K_4)^{1/4} \leq 4.1 \times 10^{-7}$ M for Ca^{2+} at saturating [ME], as deduced from the values of Table II. The standard free-energy changes associated with the binding of four Ca^{2+} in the absence and in the presence of ME, calculated as described by Keller et al. (1982), are $4\Delta G°_C = -24.8$ kcal/mol and $4\Delta G°_{C\text{-}ME} \leq -32.7$ kcal/mol, respectively. Hence, the total free-energy coupling is $4\Delta G°_{mc} \leq -7.9$ kcal/mol, and the affinity of ME for CaM is enhanced at least (1.5×10^6)-fold by saturating [Ca^{2+}].

Discussion

Previously, we reported that ME interacts with CaM and exhibits differences in both affinity and stoichiometry, depending on the presence or absence of Ca^{2+} (Comte et al., 1983). The spectroscopic study of aromatic residues (one tryptophan in ME and the two tyrosine *plus* the seven phenylalanine residues of CaM) presented here allowed a structural characterization of the interactions. Upon complex formation in the presence as well as in the absence of Ca^{2+}, the tryptophan of ME is placed in a hydrophobic environment but does not display a circular dichroic signal. Such a behavior of the tryptophan of ME has also been observed when ME interacts with hydrophobic structures such as phospholipid vesicles (Mollay & Kreil, 1973) or detergents (Lauterwein et al., 1979). At the level of the tyrosine residues of CaM, changes were observed by circular dichroism, but only in the presence of Ca^{2+}. These changes are likely to be attributed to Tyr-99 alone, since this residue is exposed at the surface of the protein, whereas Tyr-138 is shielded from the aqueous environment even in the absence of ME [for a review, see Klee & Vanaman (1982)]. We observed no changes attributable to the Phe residues of CaM; this indicates that the latter are not directly involved in the formation of the complex. The low-affinity complex formation seems not to involve any of the aromatic amino acid residues of CaM. A major difference between the high- and low-affinity complexes resides in their sensitivity to urea: the complex of ME with Ca^{2+}-free CaM is labile in 4.0 M urea; the one with Ca^{2+}-saturated CaM is resistant (Comte et al., 1983). This is also reflected at the structural level: in excess EGTA, urea abolishes the blue-shift of tryptophan fluorescence. Furthermore, circular dichroism above 250 nm (this paper) and below 250 nm (Walsh et al., 1979) showed that in the absence of Ca^{2+} urea fully denatures CaM, which, for this reason, loses its capacity for complex formation with ME.

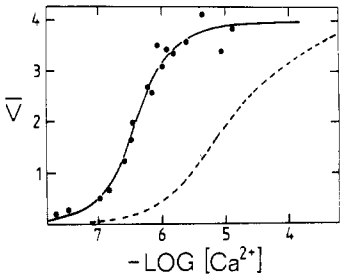

FIGURE 6: Ca^{2+} binding to CaM in the presence of ME as determined by equilibrium binding. CaM and ME both 60 μM in 0.5 mL of buffer were enclosed in a dialysis bag impermeable to both peptides and dialyzed against several concentrations of Ca^{2+} according to the experimental procedure. Closed circles are results coming from two independent experiments. (Solid line) Curve generated by the introduction of the macroscopic constants shown in Table II (third column) into the Adair equation; (broken line) Ca^{2+} binding to CaM in the absence of ME, the curve being generated by the introduction of macroscopic constants shown in Table II (second column) into the Adair equation.

Far-UV circular dichroism yielded valuable information on the secondary structure of both peptides upon interaction. Both high-affinity and low-affinity complex formation led to an increase in the α-helical content. Since under various perturbating conditions CaM displayed only decreases of the circular dichoric signal at 222 nm, whereas that of ME is enhanced in the presence of detergents, we assume that the helical content of ME alone in the complex increases drastically (from 5 to 72%) whereas the secondary structure of CaM remains virtually unchanged. This assumption is supported by the well-known properties of the two interacting peptides. Upon interaction of ME with hydrophobic structures such as membranes (Sessa et al., 1969), with detergents (Lauterwein et al., 1979), or with other melittin monomers to form a

tetramer (Brown et al., 1980; Faucon et al., 1979), a strong enhancement of its α-helical content takes place (Dawson et al., 1978; Knöppel et al., 1979). The structure of ME, determined by X-ray diffraction studies of ME grown in tetramer-promoting conditions, (Terwilliger & Eisenberg, 1982), consists of two α-helices linked by a smooth bend around the single proline in the middle of the sequence. This structure is highly amphipathic; i.e., one side along the axis of the helices is constituted of hydrophobic residues only. The hydrophobic area covered by this conformation is about 350 Å^2 (DeGrado et al., 1981). CaM on the other hand shows a small increase in α-helical content upon Ca^{2+} binding, which, if one takes into account the structural requirements of the four Ca^{2+}-binding domains, turns the protein into its maximally structured conformation, highly resistant to denaturation [for a review, see Klee & Vanaman (1982)]. CaM also exposes a Ca^{2+}-induced hydrophobic domain to the aqueous environment, as was shown by binding of fluorescent probes (LaPorte et al., 1980; Tanaka & Hidaka, 1980) and hydrophobic drugs (Levin & Weiss, 1978). This hydrophobic domain is located near tyrosine-99, which also seems to be directly involved in complex formation with ME (Head et al., 1982; Krebs & Carafoli, 1982). Hydrophobic probes inhibit competitively the CaM-induced activation of phosphodiesterase (LaPorte et al., 1980) in a similar fashion to ME. Therefore, it is likely that ME interacts with the putative interaction site of CaM for target enzymes.

In this paper, the effect of ME on the binding of Ca^{2+} by CaM was analyzed by the model of Weber (1975) for the multiple interactions of two kinds of ligands with proteins. This approach was recently employed for the characterization of the CaM–troponin I interaction (Keller et al., 1982; Olwin et al., 1982). We observed with ME the same phenomena as were noted for troponin I: first, ME enhanced markedly the affinity of the Ca^{2+} sites; second, it promoted an apparent homotropic cooperativity between these sites. The first point is readily explained by the theory of the energetics of ligand binding (Weber, 1975). Since ME and Ca^{2+} separately bind with high affinity to the "active" conformation of CaM, they displace the equilibrium toward the formation of this species and, therefore, enhance mutually the affinity for the other ligand type.

The second point can be explained by one or more of three distinct mechanisms: (1) Since free ME concentration is at a comparable order of magnitude, or lower than its dissociation constant for CaM in the absence of Ca^{2+}, it does not saturate CaM at low Ca^{2+}; when the Ca^{2+} concentration is increased, ME will bind simultaneously with the first Ca^{2+} to CaM, thus enhancing the affinity of its unoccupied Ca^{2+} sites. (2) The induction of CaM denaturation by ME prevents the binding of the first Ca^{2+} at low metal concentrations; excess Ca^{2+} reverses this situation to form the one-to-one complex with high affinity for Ca^{2+}. (3) ME provides a link between the four Ca^{2+} sites of CaM, so that the binding to one site influences the affinity of the others for Ca^{2+}; i.e., ME induces the appearance of a true homotropic cooperativity in the binding of Ca^{2+}.

The first of these three situation is certainly realized, since the K_d for ME in the absence of Ca^{2+} is higher than 10^{-5} M. The second phenomenon may also play a role, since the denaturation of CaM takes place at about 10^{-4} M ME, and the experiment was performed at 60 μM. No conclusion can yet be drawn about the third possibility, since, at any ME concentration, one of the other two mechanisms would mask it. However, one can say that the experimentally determined apparent K_d's for Ca^{2+} are higher than the actual one of the high-affinity ME–CaM complex, i.e., than the values that would be obtained if ME would not have any denaturating effect and if it saturated CaM also at low Ca^{2+} concentrations. Then, $\bar{K}_d \leq 4.1 \times 10^{-7}$ M, and $4\Delta G°_{mc} \leq -7.9$ kcal/mol. These values are much lower than those reported for the CaM–troponin I interaction ($\bar{K}_d = 1.7 \times 10^{-6}$ M and $4\Delta G°_{ic} = -5.0$ kcal/mol) (Keller et al., 1982). The overall similarity between our results and those of the CaM–troponin I system indicates that ME provides a good molecular model for a CaM-binding site. Since the α-helical conformation of ME is well described, it will be interesting to search on CaM for topological complementarities to ME. Conversely, as the knowledge on target enzymes progresses, a close look for sequence and three-dimensional structure homologies with ME may provide valuable informations on the locations and mode of action of these binding sites. Finally, the small size of ME and its high affinity make of it a good agent for competition experiments with putative CaM-stimulated enzymes and enzyme fragments.

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Registry No. ME, 37231-28-0; Ca, 7440-70-2; urea, 57-13-6; EDTA, 60-00-4.

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