The Interaction of Calmodulin with Amphiphilic Peptides*

Jos A. Cox†, Michelle Comte‡, John E. Fitton§, and William F. DeGrado∗∗

From the †Department of Biochemistry, University of Geneva, Geneva, Switzerland, the ‡Department of Biochemistry, University of Sheffield, Sheffield, United Kingdom, and the ∗∗Central Research and Development Department, E. I. du Pont de Nemours & Company, Experimental Station, Wilmington, Delaware 19898

Calmodulin has recently been shown to form exceptionally tight, calcium-dependent complexes with several natural peptides ($K_{diss} < 10^{-7}$ M). These peptides were demonstrated to be capable of forming basic, amphiphilic α-helices. To further illustrate the importance of this structural feature for calmodulin binding, several other amphiphilic α-helical peptides were tested for their ability to bind calmodulin. A monomer complex of high affinity (>10^8 M^-1), a new competition assay was devised with Sepharose 4B-conjugated melittin. Stoichiometries were assessed by electrophoresis and equilibrium size exclusion chromatography. Three peptides, which were designed to form idealized amphiphilic α-helices were tested. The basic peptides, N°-9fluorenylmethoxy carbonyl-(FMOC)-(Leu-Lys-Lys-Leu-Leu), and FMOC-(Leu-Lys-Lys-Leu-Leu-Leu-Leu), bind calmodulin in a 1:1 complex with dissociation constants of 150 and 3 nM, respectively. The acidic peptide, FMOC-(Leu-Glu-Glu-Leu-Leu-Glu-Leu), failed to bind calmodulin, even at micromolar concentrations. Complex formation between calmodulin and the 14-residue basic peptide leads to an increase in the helicity of the complex which is attributed to an increase of about 50% in the helicity of the peptide. Calmodulin also interacts with the neutral α-helical peptide toxin b-hemolysin. Concomitant with binding, the fluorescence maximum of the unique Trp residue increases 2-fold and is blue-shifted. A dissociation constant could not be unambiguously estimated though, since δ-hemolysin has a strong tendency to self-aggregate. The above data support our hypothesis that a basic, amphiphilic α-helix is a structural feature which underlies the calmodulin-binding properties common to a variety of peptides.

The striking similarities in the mechanisms of the calmodulin-mediated activation of several different enzymes (1) indicate that these enzymes possess very similar recognition sites capable of interacting with CaM. This working hypothesis has recently been reinforced by the finding that three different CaM-regulated enzymes interact with the same monoclonal antibody (2). The study of the structural features of the so-called "CaM-binding domain" is seriously hampered by the fact that 1) very few target enzymes are available in appreciable amounts in pure form; 2) most are of high molecular weight, and 3) in the case of erythrocyte (Ca2+-Mg2+)-ATPase (5) and brain adenylate cyclase, limited proteolysis of targets failed to yield fragments with a high affinity for CaM. Therefore, much interest was raised by the fortuitous finding that CaM interacts in a Ca2+-dependent way with small peptide hormones and toxins of well-known chemical structure. Some peptide hormones, such as β-endorphin and corticotropin, bind CaM albeit with affinities approximately 1000-fold lower than those of target enzymes (4). β-Endorphin forms a complex of 2 mol of hormone/mol of CaM (5), which is reminiscent of the binding of hydrophobic drugs (6, 7) and of the relevant reaction of CaM with hydrophobic probes (8).

More interestingly, a 1:1 complex of high affinity ($K_a = 3$ nM) is formed between CaM and a cytotoxic peptide from bee venom melittin (9). Concomitant with complex formation, the helical content of melittin increases from 5 to 70% (10). The α-helix formed by melittin is highly basic and amphiphilic, i.e. the hydrophobic and hydrophilic residues project from opposite faces (11, 12). Indeed, this conformation has been shown to be essential for the membrane-binding and cytotoxic effects of several lytic peptides (13, 14). Mastoparans, another group of peptide toxins which are capable of forming highly basic amphiphilic helices, were recently shown to bind CaM with nanomolar dissociation constants (15). Furthermore, a 1:1 complex with fairly high affinity ($K_a = 50$ to 140 nM) is formed between CaM and either vasoactive intestinal peptide, secretin, or gastric inhibitory peptide (16). Those homologous peptides are members of the glucagon family and share the conserved structural features of an amphiphilic helix in close proximity to basic residues (17).

Based on the above information, we hypothesize that the minimum structural requirement of a high-affinity ligand for CaM is a basic amphiphilic α-helix approximately 15 Å in length. To test this hypothesis, we studied the binding of CaM to the synthetic peptides LK1, LK2, and LE2 (LK1, FMOC-(Leu-Lys-Lys-Leu-Leu-Lys-Leu); LK2, FMOC-(Leu-Lys-Lys-Leu-Leu-Lys-Leu)z; and LE2, FMOC-(Leu-Glu-Glu-Leu-Glu-Leu-Glu-Leu)z, with FMOC being the 9-fluorenylmethoxy carbonyl group (18). These peptides form idealized basic or acidic amphiphilic α-helices. We also studied the interactions of CaM with the natural peptide δ-hemolysin, which forms neutral amphiphilic α-helices (19). Competitive assays with immobilized melittin (Melex) or with CaM-dependent cyclic nucleotide phosphodiesterase, and equilibrium
gel filtration were used as tools for the evaluation of the affinity of these peptides for CaM.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine brain CaM was purified by affinity chromatography according to the method described by Gopalakrishna and Anderson (20) followed by hydroxyapatite chromatography. "[H] Monocacyt-CaM was prepared according to Sobee et al. (21) and purified by affinity chromatography on phenyl-Sepharose equilibrium column using 2.8 ml of packed CM-Sepharose 4B (25) suspended in 0.5 M sodium borate buffer, pH 8.3, 0.5 M NaCl, 6 M guanidine-HCl. The coupling yield after 2 h at room temperature was nearly 100%. Bound melittin retained a Ca-dependent affinity for CaM, with a degree of substitution of about 0.094 mmol of melittin/mg of packed gel. Titration of Melex with radiolabeled CaM and KC1 were added to a final concentration of 1 mM and 0.6 M, respectively (based on 3020 cpm/mg of protein). Bovine brain CaM was purified by affinity chromatography on phenyl-Sepharose (see above). The coupling products between melittin and CNBr-resin by the peptides melittin, LK1, LK2, LE2, and 6-hemolysin were performed at 25°C on a Baird Atomic fluorescence spectrophotometer equipped with a 5-mm quartz cell. For titration experiments the reaction mixtures contained 30, 8, or 2 µM 6-hemolysin and increasing concentrations of CaM in 600 µl of 90 mM TES-NaOH, pH 7.0, 135 mM NaCl, 4 M urea, 1 mM CaCl2. After incubation at room temperature for 1 h, the emission fluorescence spectra were monitored after excitation at 285 nm with both slits at 5 nm. The fluorescence intensity at 350 nm was corrected for the concentration of buffer and CaM to the signal. It should be noted that in the absence of CaM, excitation of 6-hemolysin from 1 to 353 nm is accompanied by a marked blue shift of the wavelength of maximal emission (from 353 to 338 nm), indicating that 6-hemolysin is involved in monomer-polymer equilibria with Ka values in the micromolar range. However, the relative fluorescence yield at 350 nm calculated on a molar basis does not vary significantly with the concentration of the toxin in the range of 1 to 30 µM.

**Fluorescence Experiments**—Fluorescent experiments involving 6-hemolysin were performed at 25°C on a Baird Atomic fluorescence spectrophotometer equipped with a 5-mm quartz cell. For titration experiments the reaction mixtures contained 30, 8, or 2 µM 6-hemolysin and increasing concentrations of CaM in 600 µl of 90 mM TES-NaOH, pH 7.0, 135 mM NaCl, 4 M urea, 1 mM CaCl2. After incubation at room temperature for 1 h, the emission fluorescence spectra were monitored after excitation at 285 nm with both slits at 5 nm. The fluorescence intensity at 350 nm was corrected for the concentration of buffer and CaM to the signal. It should be noted that in the absence of CaM, excitation of 6-hemolysin from 1 to 353 nm is accompanied by a marked blue shift of the wavelength of maximal emission (from 353 to 338 nm), indicating that 6-hemolysin is involved in monomer-polymer equilibria with Ka values in the micromolar range. However, the relative fluorescence yield at 350 nm calculated on a molar basis does not vary significantly with the concentration of the toxin in the range of 1 to 30 µM.

**Competitive Phosphodiesterase Assay**—The enzymatic activity was assayed at 30°C by the method of Boudreau and Drummond (27); all the experimental conditions are defined by Comte et al. (9). Dissociation constants were obtained from the experimental data according to the following mathematical treatment. In the absence of peptide, the activation of phosphodiesterase is positively cooperative and may be described by the Hill equation (28) $f = \frac{[CaM]^{n} [K + [CaM]^{n}}{[K + [CaM]^{n} + [CaM]^{n}}$, where $f$ is the fraction of the maximal activation of the enzyme. Solving for [CaM] gives [CaM] = $f [K + [CaM]^{n}}/([K + [CaM]^{n})$. Now, [CaM] = $[CaM]^{n} - [CaM]^{n} - (f - [K + [CaM]^{n})$. Similarly, $[CaM] = f [P] - [CaM]^{n} - (f - [K + [CaM]^{n})$. Setting $f = (f - [K + [CaM]^{n})$ and substituting the above expressions into that for Ka, we obtain the following.

\[ Ka = \frac{([P] - [CaM]^{n}) + F - F}{[CaM]^{n} - F} \]  

The values of Ka for each peptide were calculated from the linear least square fits of this equation to the data.

**Miscellaneous**—Disc gel electrophoresis was performed on 10, 12.5, or 15% polyacrylamide gel columns in the presence or absence of 4 M urea according to the method of Head and Perry (29). Gel scanning was done with a Zeiss PMQII spectrophotometer. Peak area integration was performed by computer.

Circular dichroism spectra were recorded at 21°C with a Jasco 500 spectropolarimeter equipped with a 1-mm path length cell and the spectra were averaged four times to reduce the signal-to-noise ratio.

**RESULTS**

**Competitive Assay Using Melex**—Melittin was immobilized on CNBr-activated Sepharose 4B yielding a support (Melex) which displayed strong, calcium-dependent affinity for CaM. Fig. 1 illustrates the displacement of CaM from the Melex resin by the peptides melittin, LK1, LK2, LE2, and 6-hemolysin. The coupling products between melittin and CNBr-activated Sepharose are heterogeneous, which must be accounted for in determining dissociation constants for peptide
binding to CaM. Under the experimental conditions used, the free CaM concentration is negligibly small when compared to Melex- or peptide-bound CaM, and for a given displacement (1 - fₚ) of CaM from the resin, the relationship between Kₕ and for a model peptide (Kₕₚ) and for melittin (Kₕₘ) is Kₕₚ/Kₕₘ = [mpt]/[mel] = (Pₕₚ - CaMₘ)(1 - fₚ)/(Pₕₘ - CaMₘ(1 - fₚ)), where [mpt] and [mel] are the concentrations of free model peptide and melittin, respectively, Pₕₚ and Pₕₘ the corresponding total concentrations, and CaMₘ is the total concentration of CaM. This equation predicts that for Pₕₘ >> CaM and Pₕₚ >> CaM, if fₚ is plotted versus the log of the total peptide concentration, a family of parallel curves should be observed with a spacing of log Kₕₘ = log Kₕₚ. Indeed, LK1 and LK2 give curves parallel to that of melittin. The mean dissociation constant of CaM for LK2 was 3 nM and of CaM for LK1 150 nM, when evaluated with Equation 1 from the most significant part of the displacement curves (in the range of 25 to 65% of bound CaM). Using the fluorenyl fluorescence of LK2 and LK1, it was established that these positively charged peptides do not interact directly with the Melex resin. In contrast, the negatively charged peptide LE2 gave a dissociation curve which was clearly different from those of the other peptides because it showed strong binding to the Melex resin, i.e. under standard assay conditions, 95% of the peptide is bound to Melex at a total peptide concentration of 2.8 μM. Therefore, the affinity of the latter peptide could not be established by the Melex method.

The Melex displacement assay was also performed on δ-hemolysin (Fig. 1) but the displacement curve was irregular and could not be interpreted in terms of simple competitive behavior.

*Competitive Enzymatic Assay Using Brain Phosphodiesterase*—The fact that melittin acts as a competitive inhibitor in the activation of bovine brain phosphodiesterase by CaM was previously used as a tool for determining the affinity of melittin for CaM (9). Melittin does not interact directly with the enzyme at concentrations up to 100 nM. Applied to vaso-active intestinal peptide, this method yielded a dissociation constant of 74 nM (data not shown) in reasonable agreement with the value of 50 nM determined by Malencik and Anderson (16). This competition assay was used to assay the affinity of the peptides for CaM (Fig. 2). The peptide LE2 at 100 or 500 nM does not significantly displace the curve describing the CaM-dependent activation of phosphodiesterase, suggesting that at those concentrations no complex is formed between this peptide and CaM. This confirms our statement that in the displacement assay this negatively charged peptide acts merely as a competitor with CaM for Melex by binding to Melex. The displacement of the curve by 100 nM LK2 is similar to the one induced by an identical concentration of melittin, indicating that the two toxins have the same potency to inhibit CaM-activated processes and the same affinity for CaM, in accordance with the results of the Melex assay (see above). Similarly, 100 nM LK1 displaced the CaM activation curve, but to a lesser extent. By the data treatment described under "Experimental Procedures," dissociation constants of 250, 5, and 3 nM were calculated for LK1, LK2, and melittin, respectively. These values are in reasonable agreement with those of the Melex experiment. The experimentally determined activation curve in the presence of 100 nM δ-hemolysin was significantly different from those obtained with the other peptides, as was the case in the Melex displacement assay.

**Stoichiometry of the CaM-Toxin Complexes**—As in the case of CaM and melittin, the complex formed between CaM and LK2 is stable during electrophoresis on polyacrylamide gels in the absence or presence of urea and migrates with a mobility slightly lower than that of CaM alone. Fig. 3 (A and B) shows that at a ratio of 0.5 LK2/CaM the complex and free CaM are visible in about equal amounts (assuming equal Coomassie Blue staining), whereas at equimolar concentrations of LK2 to CaM, all CaM migrates as a complex. No
band of lower mobility than that of this complex appeared at higher amounts of LK2 up to 3 eq. When the ratio of LK2/CaM is above 2 and no urea is present, the complex band disappeared (Fig. 3A, gels 4 and 5) from the gel, probably because of the formation of positively charged aggregates.

Nonspecific complex formation has also been observed in the case of melittin (9) but is reduced, although not abolished, in 4 M urea (Fig. 3B, gels 4 and 5). The stoichiometry of the CaM-LK2 complex could be determined after a similar electrophoresis experiment followed by densitometric scanning and integration of the area of the peaks (Fig. 3C); the inflection occurs at 1.08 LK2/CaM. Fig. 3C also shows that nonspecific complex formation starts at more than 2 LK2/CaM.

The Ca dependency and stoichiometry could also be established by equilibrium gel filtration experiments in the presence and absence of 1 mM free Ca\(^{2+}\) (Fig. 4A). After fractions 16 to 27 were pooled, the stoichiometry in the pool was determined based on the ratio of fluorescence of the FMOC-containing peptide to the counts of \(^{3}H\)CaM as described under "Experimental Procedures." A value of 1.04 LK2/CaM was obtained, thus confirming the value obtained by electrophoresis. Equilibrium gel filtration further established that at 1 \(\mu\)M free LK2 there is no trace of complex formation by this negatively charged peptide with CaM (Fig. 4B).

The Ca-dependent complex formation between CaM and LK1 is shown in Fig. 5, A and B. Equilibrium gel filtration in the presence of 2 \(\mu\)M free LK1 shows that very little complex (less than 10%) is formed in 1 mM EDTA and the initial excess LK1 elutes in the total column bed volume. In the presence of 1 mM CaCl\(_2\), the CaM-containing fractions also contained approximately 0.7 mol of LK1/mol of CaM. No complex formation between LK1 and CaM could be detected by disc gel electrophoresis under conditions similar to those used in the case of LK2.

Interaction of CaM with \(\delta\)-Hemolysin—As shown in Figs. 1 and 2, \(\delta\)-hemolysin influences in a complex way the phosphodiesterase and Melex assays. In the presence of Ca\(^{2+}\), \(\delta\)-hemolysin is also retained on a column of Sepharose-conjugated CaM, but with an efficiency at least 10-fold lower than melittin (results not shown). On the other hand, disc gel electrophoresis failed to show complex formation between CaM and \(\delta\)-hemolysin. The Sephadex G-75 filtration method failed to yield a clear separation between free CaM and \(\delta\)-hemolysin, because the peptide is in an oligomeric form (19).

Fortunately, upon complex formation with CaM, \(\delta\)-hemolysin displays a two-fold enhancement of its unique Trp fluorescence as well as a blue shift of the emission maximum. Since the toxin shows a concentration-dependent blue shift of its fluorescence maximum without a significant increase of the fluorescence yield (see "Experimental Procedures"), the CaM-induced blue shift is more pronounced at 2 \(\mu\)M (from 350 to 335 nm) and 8 \(\mu\)M (from 344 to 335 nm) than at 30 \(\mu\)M (from 338 to 335 nm). At any concentration, CaM-saturated \(\delta\)-hemolysin displays the same fluorescence spectrum (maximum 335 nm) and quantum yield. Fig. 6 shows the relative enhancement at three concentrations of \(\delta\)-hemolysin as a function of the ratio of CaM to \(\delta\)-hemolysin. Apparently the CaM binds more than one molecule of the toxin. The higher apparent affinity at lower \(\delta\)-hemolysin concentration probably reflects an increase in the mole fraction of toxin molecules which are not self-aggregated and hence are available for interaction with CaM. The unusual behavior of \(\delta\)-hemolysin in the Melex and phosphodiesterase assays may also result...
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FIG. 5. Sephadex G-75 equilibrium gel filtration of the complex CaM-LK1. The experiment was performed as described under "Experimental Procedures" with 18 nmol of CaM plus 36 nmol of LK1 in the column load. The column was equilibrated in buffer containing 2 mM LK1, A, in the presence of 1 mM CaCl2. The determination of the ratio of bound LK1/CaM in the peak fraction and in the pooled fractions (horizontal bar) is as described under "Experimental Procedures." B, in the presence of 1 mM EDTA.

FIG. 6. Stoichiometric titration of 2 (○), 8 (●), or 30 (×) μM β-hemolysin with CaM based on the Trp fluorescence enhancement at 350 nm after excitation at 285 nm. The fluorescence signals were normalized assigning to the signal in the absence of CaM a value of zero, and of 1 to the signal in the presence of at least 3 eq of CaM.

from the complex competitive equilibria in which the toxin is involved.

Circular Dichroism and CaM and Its Complex with LK2—
The circular dichroism spectrum of CaM in 0.50 mM CaCl2, 5 mM Tris-HCl, pH 7.2, is illustrated in Fig. 7. Under these conditions, the mean residual ellipticity at 222 nm is −1.44 × 10⁴ deg.cm²/dmol, which is in close agreement with the value obtained at higher ionic strength (10). The helical content of CaM was estimated as 50% based on the ellipticities at 222 and 208 nm (30). Upon the addition of an equimolar concentration of LK2, the minima at 208 and 222 nm increase in intensity indicating an increase in the helical content. Since the concentration of LK2 and CaM (11 μM) are far greater than the Kd for the complex, the spectrum must reflect the complex with negligible contributions from the free components. The difference spectrum obtained by subtracting the spectrum of the complex from that of the free protein is markedly different from that of the free peptide (Fig. 7B), suggesting that LK2 or CaM change their conformations when forming a complex. It appears likely that the spectroscopic changes are due to a change in the peptide and not in CaM, because incubation of 20 μM CaM with saturating concentrations of trifluoperazine (80 μM) (a small hydrophobic drug which also interacts with the phosphodiesterase site on CaM), failed to have an appreciable effect on the circular dichroism of CaM. If the change in ellipticity associated with the binding of LK2 to CaM is due entirely to a change in the conformation of LK2, then the mean residual ellipticity at 222 nm of the bound peptide is −1.4 ± 0.1 × 10⁴ deg.cm²/dmol. This corresponds to a helical content of approximately 50% (30). In contrast, the spectra of the uncomplexed peptide are consistent with the peptide being in a random conformation in the unbound state (Fig. 7B).

DISCUSSION

Of the different methods used in this report for the evaluation of affinities between peptides and CaM, the most direct
are size exclusion chromatography and fluorometric measurement of the complex. Unfortunately, both these methods can only be used if the dissociation constant is above 100 nm. Dissociation constants for higher affinity complexes cannot be quantified by direct methods since they would require the use of nanomolar concentrations of both peptide and CaM. Even if detection methods with suitably high sensitivities were available, the experiments would be complicated by absorption of amphiphilic peptides and drugs by the tube and cell walls at such low concentrations. Therefore, we (9) and others (16, 31) used competition experiments with CaM-activated enzymes such as myosin light chain kinase or bovine brain phosphodiesterase. Furthermore, Malencik and Anderson (15) bypassed the enzymatic activity by introducing the hydrophobic probe 9-anthroylcholine as a third element in the assay. In this report, we introduced a rapid and convenient method for the evaluation of high affinity interaction involving CaM, i.e. competition of the peptide to be assayed with immobilized melittin for 3H-labeled CaM. The method yields results in reasonable agreement with those of the phosphodiesterase assay for the peptides LK1 and LK2. Polyacrylamide disc gel electrophoresis according to the method of Head and Perry (29) provides a rapid qualitative method to confirm the formation of the high-affinity complexes. The drawback of the Melex method is that it is not applicable for peptides which interact directly with the column (for instance LE2). This is not likely to be a problem, however, for basic compounds with high affinities for CaM.

Melittin (10) and LK2 (this study) adopt an amphiphilic α-helical conformation upon forming a 1:1 complex with CaM. We therefore analyzed the sequences of other CaM-binding peptides with dissociation constants of 100 nm or less. Boxed residues are the invariant hydrophilic residues. The mean of the hydration potentials (33) for the residues at a given position in the aligned sequences was calculated and plotted as a function of their position along the peptide chain. The horizontal bar corresponds to the repeat of an α-helix.

A second feature which is important for high-affinity binding to CaM is a cluster of basic residues. All the sequences illustrated in Fig. 8 have clusterings of basic residues, and the peptides with the highest positive charge density indeed bind CaM most tightly. Although charge effects appear to be of secondary importance to nonpolar effects, they contribute to the stability of the complex (32) since after a specific proteolytic cleavage of melittin at Arg 22, the affinity of the N-terminal fragment for CaM is greatly reduced. Another example is 3-hemolysin, which forms an electroneutral amphiphilic α-helix, and binds to CaM with a reduced affinity.

The model peptides LK1, LK2, and LE2 adopt random conformations in dilute aqueous solution. Indeed, all the peptides which are known to interact with CaM with a dissociation constant less than 100 nm contain a stretch of at least 12 residues which is capable of forming an amphiphilic α-helix (Fig. 8). This can be seen in their sequences as a pattern of occurrence of hydrophobic and hydrophilic residues which repeats every 3.6 residues (the repeat of an α-helix). Thus, when the CaM-binding peptides on Fig. 8, top, are properly aligned, their hydrophobic residues occupy invariant positions. Fig. 8, bottom, illustrates the mean hydration potentials of the residues in these peptides as a function of their position along the chain. If the first two residues are omitted, a curve is observed which approximates a sine wave with a repeat of 3.6 residues. Thus a sequence capable of forming an amphiphilic α-helix is an invariant feature among these peptides.

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Fig. 8. The amino acid sequences of peptides which interact with CaM with dissociation constants of 100 nm or less. Boxed residues are the invariant hydrophilic residues. The mean of the hydration potentials (33) for the residues at a given position in the aligned sequences was calculated and plotted as a function of their position along the peptide chain. The horizontal bar corresponds to the repeat of an α-helix.
but form helices upon interaction with a variety of hydrophobic surfaces (13). On the other hand, LK1 is too short to form stable helices and binds poorly to hydrophobic surfaces. LK2 binds CaM with an affinity which is as high as that of melittin, and complex formation coincides with an increase of the helical content. As expected, LK1 had a 100-fold poorer affinity for CaM, comparable to the affinity of CaM for \( \beta \)-endorphin and corticotropin (4). Finally, LE2, which forms acidic amphiphilic helices, showed no interaction with CaM. Taken together, these data show that a minimal structural requirement for a high-affinity CaM-binding peptide is a basic, amphiphilic \( \alpha \)-helix of at least three turns in length. The precise geometries of the side chains projecting from the helix are of little importance as long as the basic amphiphilic structure is maintained.

\( \delta \)-Hemolysin has a surprisingly low affinity for CaM. This peptide forms neutral, amphiphilic helices and has a lytic potency similar to that of melittin (23). The main structural difference between \( \delta \)-hemolysin and the above peptides is that \( \delta \)-hemolysin is in a helical, aggregated form under our experimental conditions. If we assume that nonpolar interactions are the driving force for both aggregation and complexation with CaM, then the net stabilization of the complex depends on the state of aggregation of the unbound peptide. Clearly, competition between self-aggregation and complexation should decrease the apparent affinity of \( \delta \)-hemolysin for CaM.

Complete elucidation of the precise details of the interaction of peptides with CaM will require further physical and chemical studies. However, we can currently speculate on the mode of interaction based on the known structures of calcium-binding proteins. By homology to parvalbumin, it has been hypothesized that CaM is composed of four structural units each responsible for binding one calcium (for a recent review, see Ref. 1). These structural units are referred to as EF hands and each unit contains a helix on either side of a calcium-binding loop. The amino-terminal helix in each of these units (referred to here as the E helix) is highly acidic and amphiphilic in the sequence of CaM (Fig. 9). Further, the first and third E helices in the CaM sequence contain several hydrophobic residues which are partially solvent-exposed if the folding of CaM is similar to that of vitamin D-dependent calcium-binding protein or parvalbumin. We propose that basic amphiphilic \( \alpha \)-helical peptides bind to CaM by associating with one of the E helices. The most likely orientation of the basic helix would be antiparallel to the E helix. The driving force would be dehydration of the hydrophobic residues as well as electrostatic attractions between the helices.

In conclusion, we have shown that a basic amphiphilic \( \alpha \)-helix is a structural commonality underlying the CaM-binding properties of a variety of peptides. The basic helix is hypothesized to associate with one of the four proposed E helices in CaM. The precise location and characterization of the site is the subject of subsequent investigations.

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**APPENDIX**

**Determination of Dissociation Constants from Peptide-induced Calmodulin Dissociation from Melex**

We assume that Melex contains \( n \) classes of independent CaM-binding sites \( (b) \), each arising from a different coupling product between melittin and CNBr-Sepharose. The dissociation constant \( (K_i) \) for the binding of CaM to a class of sites \( b \), is given by \( K_i = [\text{CaM}](b)/[\text{CaM}.b] \). The curves for the peptide-induced displacement of CaM from a CaM-saturated Melex resin cover only 2 log units from the first detectable displacement to complete displacement. Thus, the various sites have similar affinities for CaM. Then, when the concentration of the total number of site \( b_r \) is in large molar excess over CaM, the concentration of a given type of free site \( b \) is approximated by the total concentration of this site. The dissociation constant can now be expressed as:

\[
K_i = \frac{[\text{CaM}]}{[\text{CaM}.b]}
\]

where \( a_i \) is the fraction of the total sites which are of the \( i \)th type and \( b_r \) is the total concentration of CaM-binding sites on Melex. The dissociation constant for a peptide (P) which is competing for the Melex-bound CaM is \( K_p = [\text{CaM}]/[\text{CaM}.P] \), since the concentration of free CaM is much less than peptide-bound or Melex-bound CaM, \( K_p = [\text{CaM}]/[\text{CaM}.P] \), where \( [\text{CaM}.P] \) is the total CaM concentration and \( f_i \) is the molar ratio of Melex-bound to total CaM concentrations.

Solving for [CaM] and substituting into Equation A1 gives:

\[
K_p = \frac{K_p[\text{CaM}][P]}{[\text{CaM}.P]}
\]

The concentration of Melex-bound CaM is calculated by solving for [CaM].b and summing over all \( n \) classes of sites.

\[
\sum_{i=1}^{n} [\text{CaM}.b_i] = [\text{CaM}] [\text{CaM}] [\text{CaM}]
\]

\[
K_p = \frac{K_p[\text{CaM}][P]}{[\text{CaM}.P]}
\]

Now, for two peptides, \( P_1 \) and \( P_2 \), having dissociation constants \( K_{p1} \) and \( K_{p2} \), the ratio of \( K_{p1} \) to \( K_{p2} \) can be calculated by determining the concentrations of \( P_1 \) or \( P_2 \) needed to displace the same amount of calmodulin from Melex. Combining Equation A3 for the two peptides gives:

\[
\frac{K_{p1} \sum_{i=1}^{n} (a_i/K_i) [P]}{1 + K_{p1} [b_r] \sum_{i=1}^{n} (a_i/K_i) [P]} = \frac{K_{p2} \sum_{i=1}^{n} (a_i/K_i) [P]}{1 + K_{p2} [b_r] \sum_{i=1}^{n} (a_i/K_i) [P]}
\]

The concentration of a free peptide \([P]\) is related to the total...
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concentration of the peptide $P_T$ by the equation $[P] = [P_T] - [CaMT]$ (1 - $f_b$). Thus, if $K_{pl}$ is known, $K_{pl}$ can be calculated.

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