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The Design, Synthesis, and Characterization of Tight-Binding Inhibitors of Calmodulin

William F. DeGrado, Frank G. Prendergast, Henry R. Wolfe, Jr, and Jos A. Cox

E.I. du Pont de Nemours & Company, Central Research & Development Department, Experimental Station, Wilmington, Delaware 19898 (W.F.D., H.R.W., Jr.), Department of Pharmacology, Mayo Foundation, Rochester, Minnesota 55905 (F.G.P.), and Department of Biochemistry, University of Geneva, Geneva, Switzerland (J.A.C.)

Based on a consideration of the probable structure of calmodulin and some natural peptides known to interact with it, two calmodulin-binding peptides were designed. These peptides bind to calmodulin in helical conformations and are capable of forming electrostatic and hydrophobic interactions with calmodulin. Their dissociation constants for binding ($\leqslant$210 and 400 pM) place them as the tightest-binding inhibitors of calmodulin thus far reported. The study of the interactions of these and similar peptides with calmodulin will provide valuable insights into the mechanisms whereby calmodulin binds to target enzymes, and it also serves as an excellent model system for exploring the physical chemistry of protein-protein interaction.

Key words: peptide design, calmodulin-peptide interactions, calmodulin

Calmodulin is a small, acidic protein that interacts in a calcium-dependent manner with a number of regulatory enzymes including cyclic nucleotide phosphodiesterase, myosin light chain kinase, and certain types of adenylate cyclases [1]. The elucidation of the structural basis for calmodulin's interactions with these and other target enzymes has been hampered by their large size and unknown three-dimensional structures. Calmodulin also binds a number of hydrophobic drugs competitively with respect to these target enzymes [2]. The study of the interactions of calmodulin with these compounds provides a good model system for studying the structural requirements for enzyme binding and activation. The first reported inhibitors of calmodulin were basic, hydrophobic drugs of the phenothiazine family [3]. The phenothiazines bind to calmodulin in the presence of calcium with a stoichiometry of two drug molecules per calmodulin and they have dissociation constants in the micromolar

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range. The primary driving force for the binding of these drugs appears to be hydrophobic in nature [4]. Apparently, a hydrophobic site is formed on calmodulin subsequent to interaction of the protein with calcium ions, and it is to this site that apolar molecules adsorb [5]. Unfortunately, there also seems to be several calcium-dependent, nonspecific sites on calmodulin with only slightly lower affinities for these low-molecular-weight hydrophobic compounds, and this feature limits their usefulness as structural probes [6].

More interestingly, calmodulin has recently been found to have an exceptionally high affinity for a number of peptides [7–11]. Typically, complex formation is calcium dependent, and two peptides are bound per calmodulin when the concentration of the peptide is in excess over that of calmodulin [12]. However, in many cases, one peptide-binding site on calmodulin has a much higher affinity than the other, so that under a variety of conditions, a 1:1 complex is the only species detected [13,7–11]. Peptides reported to form tight complexes represent several distinct functional classes including opiate peptides such as β-endorphin [12] and dynorphin [11], various members of the glucagon family [8,9], and cytotoxic peptides including the mastoparans [10,11] and melittin [13]. These peptides vary considerably, not only in their amino acid sequences, but also in their chain lengths, which range from 14 to over 40 residues. It thus appears that calmodulin must recognize general structural features common to these peptides rather than a specific amino acid sequence.

One feature common to the amino acid sequences of all the calmodulin-binding peptides is a cluster of residues that are positively charged at neutral pH [8,9,11]. These residues appear to make an electrostatic contribution to binding, as calmodulin is a highly acidic protein. This is consistent with the observed dependence of ionic strength on peptide binding; increasing ionic strength decreases the affinity of dynorphin, a highly basic peptide, for calmodulin. However, electrostatic interactions alone are not sufficient to account for the binding of the peptides, as polylysine does not interact with calmodulin [8].

Another structural feature postulated to be required for binding is an amphiphilic α-helix, a structure in which hydrophobic and hydrophilic residues segregate on opposite faces of the helix [7,14]. At first, evidence along these lines was circumstantial; all the peptides known to interact with calmodulin with submicromolar dissociation constants contained sequences capable of forming amphiphilic α-helices [7,14], and circular dichroism spectroscopy indicated that the helical content of β-endorphin [15], mastoparans [14], and melittin [16] increased when these peptides bound to calmodulin. Furthermore, using deletion peptides derived from the sequence of β-endorphin, Puett and co-workers [17] demonstrated that the calmodulin-binding region of this peptide corresponded to a sequence that had previously been demonstrated to form an amphiphilic α-helix [18].

To demonstrate more directly the role of charge and amphiphilicity in calmodulin binding, we synthesized several model peptides (I-III), which were designed to contain the quintessential elements of basic and acidic amphiphilic α-helices [7].

I *FMOC(LeuLysLysLeuLeuLysLeu)$_1$
II FMOC(LeuLysLysLeuLeuLysLeu)$_2$
III FMOC(LeuGluGluLeuLeuGluLeu)$_2$

*FMOC = N-α-fluorenylmethyloxycarbonyl.

Peptide II is capable of forming four turns of α-helix in which leucyl and lysyl residues occur on opposite faces of the helix. This peptide bound calmodulin competitively with respect to phosphodiesterase in a calcium-dependent manner with a 3 nM dissociation constant. This dissociation constant is identical to that found for melittin [13], the most potent natural inhibitor of calmodulin [11]. It is also well within the range observed for calmodulin binding to its target enzymes (10^{-10}–10^{-9} M) [1]. In contrast, peptide I, which has a much lower helical potential owing to its decreased length, bound calmodulin with reduced affinity (0.15 μm). The negatively charged analog, III, failed to bind calmodulin, illustrating the charge requirements for binding.

These results suggested that basic, amphiphilic peptides bind to an acidic, hydrophobic region on calmodulin. To identify this region we constructed a three-dimensional structure for calmodulin [19], starting from the backbone atom coordinates of the homologous protein of known crystal structure, intestinal calcium-binding protein (ICB) [20]. Calmodulin contains two internally homologous domains, both of which are homologous to ICB. Models for each of these domains were constructed using interactive computer graphics to replace the side chains of ICB with those of either domain while maintaining the position of the main chain atoms invariant. The geometries of the resulting structures were optimized by energy refinement. The two domains were then combined by making the last helix of the first domain contiguous with the first helix of the second domain. The resulting model for calmodulin contains one shallow hydrophobic surface flanked by a region of highly concentrated negative charge. These regions are likely candidates for peptide-binding sites. The site on the second domain had the most negative electrostatic potential, so it has tentatively been assigned as the high-affinity peptide-binding site. Biochemical studies on the isolated domains of calmodulin, obtained by limited proteolysis, also showed that only the second domain was capable of binding certain target enzymes [21] and that this domain had the highest affinity for the basic, amphiphilic α-helical peptide mastoparan X [11].

Computer modeling [19] of the interaction of the second domain of calmodulin with peptide I indicated that a reasonable complex could indeed by formed, and it helped to further define the structural requirements for peptide binding. The length of the hydrophobic patch on the second domain of calmodulin appeared to be shorter than the length of peptide II when the latter was in a helical conformation. Immediately adjacent to this patch were several acidic residues that were proximal to the leucyl side chains near the N-terminus of the peptide. It seemed likely that if a lysine residue were introduced onto the otherwise uninterrupted hydrophobic face of the peptide helix, the affinity of the peptide for calmodulin might be enhanced. To test this, we synthesized peptides IV and V, the sequences of which are compared with peptide II in Figure 1. Peptides IV and V differ from peptide II in having a tryptophan at position 3 as a fluorescent probe in their sequences rather than a fluorenyl group

Peptide II FMOC Leu Lys Lys Leu Leu Lys Leu Leu Lys Lys Leu Leu Lys Leu

Peptide IV Leu Lys Trp Lys Lys Leu Leu Lys Leu Leu Lys Lys Leu Leu Lys Leu Gly

Peptide V Lys Leu Trp Lys Lys Leu Leu Lys Leu Leu Lys Lys Leu Leu Lys Leu Gly

Fig. 1. The amino acid sequences of peptides II, IV, and V.

(FMOC). Mastoparans contain tryptophan at homologous positions in their sequences, so it was anticipated that a Trp would not disrupt calmodulin binding in peptides IV and V.

Peptides IV and V also contain two extra residues at their amino terminus and an extra glycine at the C-terminus, which was added for synthetic convenience. The slight increase in chain length should favor helix formation [22] thus improving calmodulin binding. These peptides differ from each other by having an amino terminal sequence of Leu-Lys-Trp in peptide IV but Lys-Leu-Trp in peptide V. The result of this change is that peptide V can form an uninterrupted amphiphilic α-helix, while peptide IV contains a lysyl residue on the otherwise totally hydrophobic side of its helix. In this paper we describe the determination of the affinities of these peptides by competition assays [7] using phosphodiesterase or immobilized melittin, and we characterize the complexes formed by these peptides with calmodulin by circular dichroism (CD) and fluorescence spectroscopy. Both peptides bind calmodulin stronger than any peptide previously reported in the literature. Further, in agreement with our expectations, peptide IV, which cannot form an uninterrupted amphiphilic α-helix, binds calmodulin more tightly than peptide V.

MATERIALS AND METHODS

Materials

Bovine brain calmodulin used in all studies except the fluorescence work was purified by the procedure of Gopalakrishna and Anderson [23], followed by hydroxylapatite chromatography. For fluorescence work calmodulin was purified as described previously [24]. Peptides IV and V were synthesized by the Merrifield [25] solid-phase method, starting with a chloromethyl polystyrene 1% divinylbenzene resin. Protecting groups and the synthetic protocol were the same as those described previously [26]. The peptides were cleaved from their support by treatment with HF/anisole (9:1) at 0°C for 45 min. The crude products were purified in a single step by reversed-phase HPLC using a PRP1 (Hamilton) semipreparative column with a linear gradient of 20–50% CH_3CN containing 0.1% aqueous trifluoroacetic acid varying at 0.5%/min. (flow 4 ml/min). The purified products were >95% pure based on amino acid analysis, reversed-phase HPLC, TLC, and fast atom bombardment mass spectroscopy. The molar extinction coefficients at 280 nm were 5,500 $\pm$ 200 $M^{-1}cm^{-1}$ as determined by amino acid analysis.

Disk gel electrophoresis was performed in the presence of 1 mM $CaCl_2$ or EDTA [27]. Competitive immobilized melittin (Melex) and phosphodiesterase assays were carried out as described previously [7]. Fluorescence and circular dichroism measurements were performed exactly as described previously [7,14].

RESULTS

Formation and Stoichiometry of Calmodulin-Peptide Complexes

As was the case for melittin [13] and peptide II [7], peptides IV and V formed calcium-dependent complexes with calmodulin that were stable during electrophoresis and had only slightly lower mobilities than calmodulin alone. Figure 2A, lane 1, shows the position at which calmodulin migrates in the absence of added peptides. When peptide IV is added at a ratio of 0.5 peptide IV/calmodulin (lane 2), the

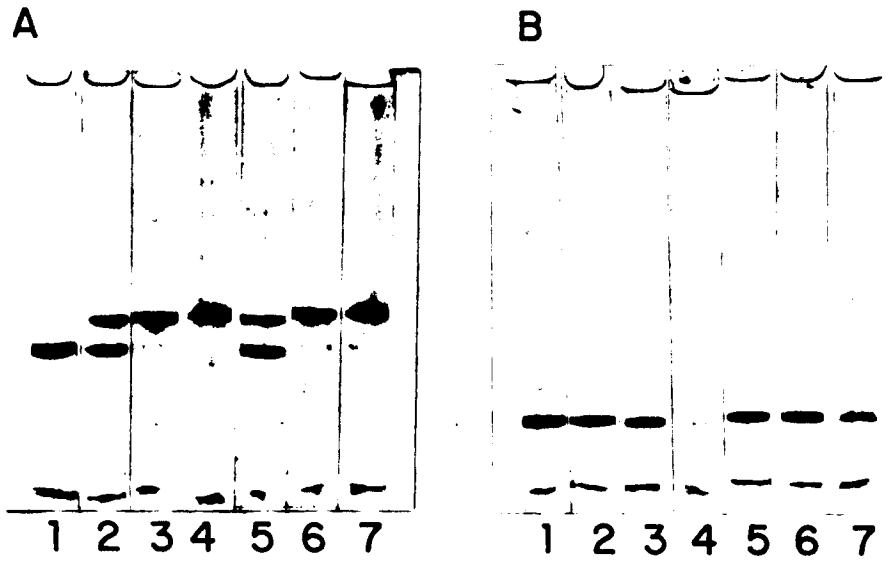

Fig. 2. Complex formation between calmodulin and peptide IV and V monitored by 12.5% polyacrylamide gel disc electrophoresis in the presence of 1 mM $CaCl_2$ (A) or 1 mM EDTA (B). Free peptide IV or V do not migrate in this electrophoretic system. All samples contain 0.18 nmol of calmodulin: (1) calmodulin alone; (2) 0.09 nmol IV; (3) 0.18 nmol IV; (4) 0.54 nmol IV; (5) 0.09 nmol V; (6) 0.18 nmol V; (7) 0.54 nmol V.

calmodulin band and a new band, which is attributable to the complex, appear in approximately equal intensities. At equimolar ratios of peptide/calmodulin only the band owing to the complex is observed (lane 3). No bands of lower mobility than that of this complex appeared when higher amounts of peptide IV, up to 3 eq, were added, although the intensity of this band decreased somewhat. Similar behavior has been observed previously for melittin and peptide II [7] and may be attributed to the formation of positively charged aggegates owing to nonspecific interactions between the basic peptides and calmodulin. The behavior of peptide IV and calmodulin in the absence of calcium supports this interpretation. When calmodulin was incubated with increasing amounts of peptide IV in the presence of 1 mM EDTA (Fig. 2b, lanes 1–4), no bands attributable to a complex were formed, although the decrease in the intensity of the calmodulin band was even more pronounced than in the presence of calcium, once again suggesting the formation of nonspecific aggregates that failed to enter the gel. Thus, calmodulin appears to contain a single high-affinity calcium-dependent site for peptide IV, and calcium-dependent, nonspecific sites of lower affinities. Very similar results were obtained for peptide V (Fig. 2).

Determination of the Affinities of Peptides IV and V for Calmodulin

Recently, we introduced Sepharose 4B-conjugated melittin (Melex) [7] as a convenient reagent for determining calmodulin's affinity for peptides. Melex has a very high calcium-dependent affinity ($K_d \sim 3$ nM) for calmodulin. Figure 3 illustrates the displacement of calmodulin from the Melex resin induced by the peptides IV, V, and melittin. The displacement curves are related to the dissociaton constants of the peptides for calmodulin by the following equation:

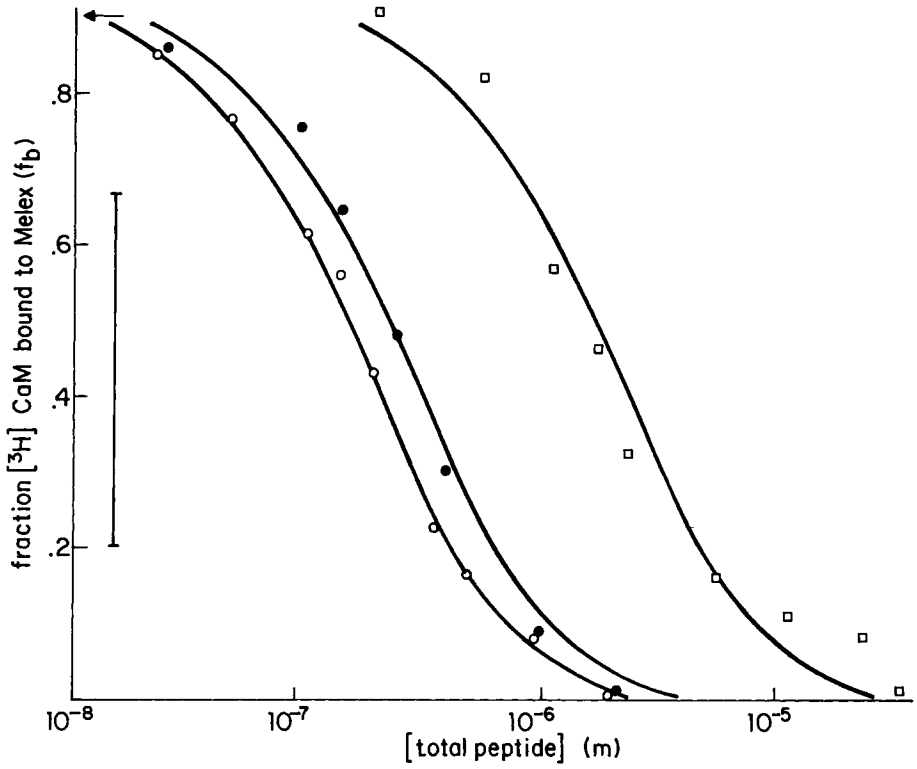

Fig. 3. Displacement of Melex-bound [^{3}H]CaM by three peptides. The incubation medium contained Melex to a final concentration of 4 μM functional, immobilitzed melittin units, 99 nM CaM, trace amounts of soluble melittin (□), IV (○), or V (●). The amount of Ca-sensitive complex between CaM and Melex was determined after release with EDTA as described previously [7]. The vertical bar corresponds to the zone of f_b where the determination of the K_d with Equation 1 yields reproducible results.

$$K_{pep}/K_{mel} = ([P_{pep}] - [CaM_T](1 - f_b))/([P_{mel}] - [CaM_T](1 - f_b)), \quad \text{(Eq. 1)}$$

where $[P_{mel}]$ and $[P_{pep}]$ are the total concentrations of melittin and peptide IV or V required for a given fractional displacement $(1 - f_b)$ of calmodulin from the resin, and CaM_T is the total calmodulin concentration. From the known dissociation constant of 3 nM for K_{mel}, values of 210 ± 20 pM and 390 ± 20 pM were calculated for the dissociation constants of peptides IV and V, respectively. Within experimental error identical dissociation constants were calculated irrespective of the value of f_b at which they were determined. The good agreement between the experimental data and the theoretical mass action treatment indicate that only 1:1 complexes are involved in dissociation of calmodulin from the resin. Furthermore, the fact that peptides IV and V had greater affinities for calmodulin than melittin could be confirmed by the competitive phosphodiesterase assay (data not shown). However, this assay is inherently inaccurate for determining the dissociation constants of peptides whose affinities greatly exceed phosphodiesterase's affinity for calmodulin (3 nM). In contrast, the

Melex assay can be used to determine subnanomolar dissociation constants, since the concentration of the competing ligand (Melex) can be added in molar excess over the inhibitory peptide, thus allowing the dissociation constant to be determined from the partition ratio.

Fluorescence Studies

Fluorescence spectroscopy [8,9,11,14] has proven to be a powerful method for probing the interactions of tryptophan-containing peptides with calmodulin, which does not contain tryptophan. When mastoparan X or *Polistes* mastoparan, both of which contain a single tryptophanyl residue, form complexes with calmodulin, their fluorescence emission maxima (λ_{em}[max]) shifts from approximately 350 to 325 nM, their fluorescence anisotropies increase markedly, and the second-order rate constant (k_q) for collisional quenching of their tryptophanyl fluoresence by acrylamide decreases five- to tenfold [14]. Taken together, these shifts indicate that when the peptides form complexes with calmodulin, the indole ring of their tryptophanyl residues are partially immobilized in an apolar environment [14]. Peptides IV and V show even greater shifts in their fluorescence properties upon complex formation. The value of λ_{em}(max) is shifted from 350 to 321 nM for each peptide (Fig. 4), and the anisotropy concomitantly increases from 0.03 to 0.12. The degree of accessibility of the tryptophanyl residues to small molecules, as measured by acrylamide quenching, is particularly low for the complexes of IV and V with calmodulin, having k_q values of 0.24×10^9 $M^{-1}sec^{-1}$ and 0.28×10^9 $M^{-1}sec^{-1}$, respectively (Fig. 5). These are the lowest values thus observed for calmodulin-binding peptides (Table I) . The fluorescence properties of IV and V are comparable to those found in ribonuclease T_1, a protein that contains a single tryptophan. Examination of the structure of this enzyme (Saenger W, personal communication) indicates that its tryptophan is almost completely inaccessible to a probe the size of a water molecule. The values of λ_{em}(max) and k_q for ribonuclease T_1 are 319 nM and 0.20×10^8 M^{-1}, respectively. Thus, the fluorescence data for IV and V are consistent with their tryptophanyl residues being directly involved in complex formation and being relatively solvent-inaccessible in the time-averaged structures of the complexes.

Circular Dichroism of Calmodulin-Peptide Complexes

Circular dichroism has been extensively used to show that various peptides become more helical upon interacting with calmodulin [7,14,17]. Circular dichroism spectroscopy of IV and V indicates that they are in random conformations at 10 μM concentration in 10 mM Tris HCl, pH 7.3 ($\theta_{222} = -3{,}000$ deg cm^2/dmol). When equimolar amounts of IV or V are added to calmodulin, the minima at 222 and 208 nM in calmodulin's spectrum increase in magnitude. The difference in ellipticity, if attributed entirely to the peptide, corresponds to a value of $\theta_{222} = -20{,}000 \pm 2{,}000$ deg cm^2/dmol. This represents a helical content of approximatley 65% when calculated as in reference [28].

CONCLUSIONS

Peptides IV and V have 15- and 7.5-fold greater affinities than melittin for calmodulin, placing them as the most potent calmodulin inhibitors thus far reported. The dissociation constants for IV and V are calculated to be 210 and 390 pM,

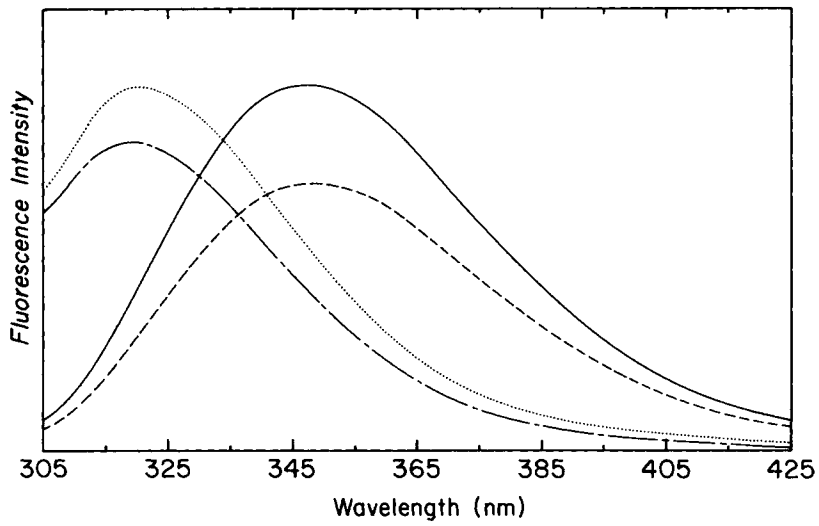

Fig. 4. Corrected tryptophan emission spectra for peptides IV and V free in aqueous solution (——) and (– – –), respectively, and bound to bovine brain calmodulin (— — —) and (· · · · ·), respectively. Spectra were taken at 25°C. The peptides and protein-peptide complexes were dissolved in 50 mM NaCl and 1 mM $CaCl_2$ buffered to pH 7.0 with 20 mM N-tris [Hydroxymethyl]-Z-aminoethane-sulfonic acid (TES). Excitation wavelength was 295 nm with a 2-nm bandpass, while the emission bandpass was 1 nm. The different spectral intensities do not reflect differences in quantum yield and are meant only to display relative spectral location. Tryptophan fluorescence emission wavelengths maxima were 348 nm and 349 nm for IV and V, respectively (free in aqueous buffer), and 320 nm for both peptides bound to calmodulin.

respectively, using our value of 3 nM for the dissociation constant of the melittin/calmodulin complex [7]. Under somewhat different conditions, Malencik and Anderson [11] have calculated a value of approximately 50 pM for the dissociation constant of the melittin/calmodulin complex. Assuming the relative affinities of peptides IV, V, and melittin remain the same under these conditions (0.1–0.2 M KCl in the absence of serum albumin), we would predict dissociation constants as low as 7 and 13 pM, respectively, for the synthetic derivatives.

Peptides IV and V also bind calmodulin approximately 7.5- and 15-fold more tightly than peptide II. This is probably due to the slightly longer chain length (and hence increased helix stability) for IV and V vs II. Also, the replacement of leucine for tryptophan at position 3 of their aligned sequences (Fig. 1) is expected to increase the stability of the complex owing to the increase in hydrophobicity. A more easily

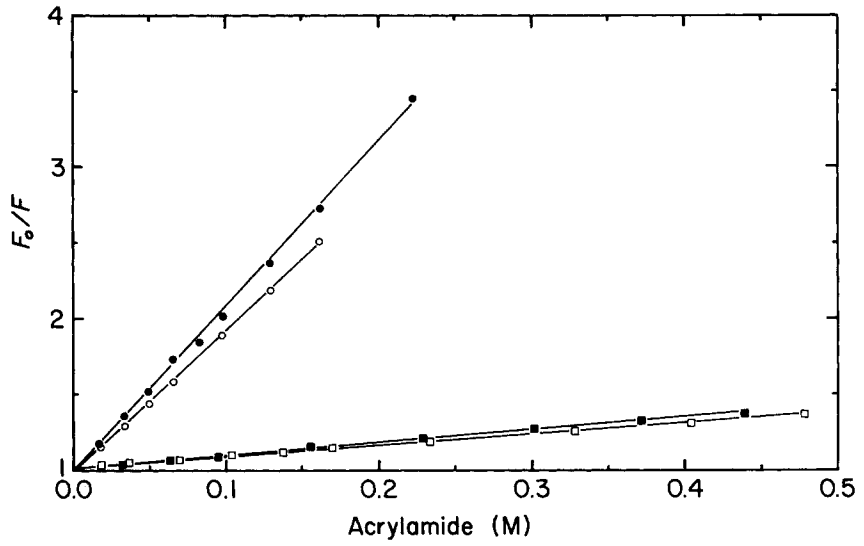

Fig. 5. Stern-Volmer plots of acrylamide quenching of tryptophan fluorescence of IV and V free in aqueous solution (—○—○—) and (—●—●—), respectively, and bound to bovine brain calmodulin (—■—■—) and (—□—□—), respectively. Solution conditions were as described in Figure 4 above. The slopes of acrylamide quenching of the tryptophan fluorescence of the bound peptides are more than a factor of ten smaller than those for the free peptide. These differences in the slopes are reflected in the calculated biomolecular quenching constants (Table I).

TABLE I. Acrylamide Quenching Constants for Tryptophan Fluorescence of Free Peptides and Peptides Bound to Calmodulin

Peptide or peptide/CAM complex	$k_q \times 10^{-9}\ M^{-1}\ sec^{-1}$
Mastoparan X (MX)	3.9
Polistes-mastoparan (PM)	3.5
Peptide IV	3.1
Peptide V	3.5
MX-CAM	0.39
PM-CAM	0.60
IV-CAM	0.24
V-CAM	0.28
Ribonuclease T_1	0.30

interpreted difference in affinities is the twofold enhanced affinity of IV vs V. Peptide IV has a lysine residue on the otherwise hydrophobic side of its amphiphilic helix. It was anticipated that a lysine in this position might have electrostatic interactions with negatively charged residues in the last E-helix of calmodulin's second domain. Indeed, this change is not only tolerated, but also actually enhances binding twofold when compared to peptide V, which has a leucine at this position. This small effect represents a difference of 0.4 kcal/mol, not an unreasonable number for an electrostatic interaction between charged groups that are largely exposed to an aqueous environment and counterions.

The premise for studying the interactions of amphiphilic peptides with calmodulin is that the same types of interactions might be operational in the interaction of

calmodulin with its target enzymes. Very recently, this premise has been given credence by the isolation of a 27-residue peptide that appears to house the calmodulin binding domain of myosin light chain kinase [29]. This peptide was isolated from a CNBr digest of the enzyme and binds to calmodulin at least as tightly as does the intact enzyme. The peptide is highly basic, and by the Edmondson helical wheel projection, it can be shown to form an approximately 15-residue amphiphilic α-helix.

Calmodulin-binding peptides also provide an excellent model system for understanding how other amphiphilic peptides interact with their receptors. Recently, the amphiphilic α-helix has been shown to be an important feature required for the binding of peptide hormones to their receptors [30]. Calmodulin-binding peptides provide excellent models for elucidating the structural basis of these interactions, for, unlike hormone receptors, which often contain multiple protein, lipid, and carbohydrate components, calmodulin is a small protein whose crystal structure has been determined [31]. In addition, the gene for calmodulin has been cloned and expressed at high levels in *Escherichia coli* [32,33]. By site-directed mutagenesis it is possible to change residues at will in calmodulin. Thus, it is possible to systematically vary the sequence of both a peptide and its receptor in an effort to determine how electrostatic, van der Waals, and "hydrophobic" interactions stabilize protein-protein interactions. Further, the marked changes in the fluorescence spectrum, anisotropy, and lifetimes of the tryptophan residue on the apolar face of the amphiphilic helix provide a valuable tool for investigating peptide/calmodulin interactions under kinetic or equilibrium conditions. This approach also provides a model for studying how the tryptophan fluorophore's environment determines properties such as fluorescence lifetimes, anisotropies, and energy transfer. Lastly, the relatively small size of the peptides and degeneracy in possible amino acid sequences allow for ready incorporation of spectroscopic probes, especially for NMR studies. The system is presently being exploited along all these avenues.

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