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Probable Role of Amphiphilicity in the Binding of Mastoparan to Calmodulin†

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ABSTRACT: Two-dimensional helical wheel diagrams and calculations of mean hydrophobic moments show mastoparan, mastoparan X, and *Polistes* mastoparan to have all the properties expected for amphiphilic helices. Circular dichroic properties are consistent with a random form for these peptides in dilute aqueous solution, but >50% helix is apparent when the peptides are dissolved in 70% trifluoroethanol/water mixtures (v/v) or when the peptides are bound to calmodulin. Changes in the fluorescence spectra, anisotropy, and accessibility of tryptophan whose indole side chain is on the apolar surface of the amphiphilic helix imply a significant role for the apolar surface in the binding of the mastoparans and another amphiphilic peptide, melittin, to calmodulin. These data provide a useful model for designing high-affinity synthetic peptide inhibitors of calmodulin.

Several small peptides have been found recently to bind with high affinity to calmodulin and to inhibit expression of function in this protein (Weiss et al., 1980; Malencik & Anderson, 1982, 1983a,b, 1984; Giedroc et al., 1983; Comte et al., 1983; Maulet & Cox, 1983). Melittin, a 26 amino acid peptide, and mastoparans, a group of three tetradecapeptides (see Figure 1 for sequences), have the highest affinities of the peptides studied to date. The dissociation constants for the complexes of calmodulin with mastoparan, mastoparan X, and *Polistes*

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mastoparan were determined to be, respectively, 0.3, 0.9, and 3.5 nM by competitive dissociation of the calmodulin–myosin light chain kinase complex (Malencik & Anderson, 1983b). For the calmodulin–melittin complex, a dissociation constant of 3 nM was reported on the basis of equilibrium binding experiments (Comte et al., 1983), but Malencik & Anderson (1984) estimated a much lower value of 0.1 nM (or lower) for this interaction. It is clear from these studies that melittin and the mastoparans have calmodulin affinities at least as high as (and probably higher than) some of the enzymes that calmodulin stimulates. Consequently, these peptides are attractive models for studying the structural requirements for binding of such enzymes to calmodulin.

Circular dichroism (CD)[1] data have suggested that a substantial degree of α-helical structure is induced in melittin upon binding to calmodulin (Maulet & Cox, 1983). This interaction is also accompanied by an 8-nm blue shift in the tryptophan fluorescence emission from melittin (Maulet & Cox, 1983). Malencik & Anderson (1983b, 1984) have observed even larger blue shifts for the emission spectra of the single tryptophan in both mastoparan X and *Polistes* mastoparan upon interaction with calmodulin. There were concomitant changes in the tryptophan fluorescence anisotropy. These authors have suggested that a strongly polar tripeptide sequence three residues away from a pair of hydrophobic residues in these and some other peptides may serve as a common recognition sequence for calmodulin (Malencik & Anderson, 1982, 1983a). In other words, some periodicity in the peptide structure appears to be important in interaction with calmodulin, but the basis for the structural recognition of mastoparans by this protein is not clear at the present time.

Recent work by Higashijima and co-workers (Higashijima et al., 1983) has shown that mastoparan forms a "largely" (60%) α-helical conformation in neat methanol but is considerably less ordered in aqueous solution. These workers showed in addition that binding of the peptide to phospholipid vesicles results in an increase in helicity. Melittin is known to have an essentially random form in dilute aqueous solution but becomes helical (>70%) when it interacts with phospholipid surfaces (Dufourcq & Faucon, 1977; Dawson et al., 1978), with detergents (Lauterwein et al., 1979), or with calmodulin (Maulet & Cox, 1983) or when dissolved either in organic solvent/water cosolvents (F. G. Prendergast and co-workers, unpublished results) or in media of high ionic strength (Drake & Hider, 1979). Edmundson's two-dimensional helical wheel diagrams in which all the amino acid side chains are viewed along the principal axis of the putative helix (Schiffer & Edmundson, 1967) have been used by Kaiser, Kézdy, and co-workers to show that the sequence of melittin favors formation of an amphiphilic helix (Degrado et al., 1981). More recently, Eisenberg and co-workers have verified the amphiphilic character of melittin by calculation of hydrophobic moments (Eisenberg et al., 1982). The high affinity of the mastoparans and melittin for binding to calmodulin suggests a commonality in the driving force for complex formation. The most obvious possibility is that the mastoparans are also amphiphilic and that *this* may be a major determinant for peptide–calmodulin interaction. Hydrophobic moment calculations (Eisenberg et al., 1982; Sweet & Eisenberg, 1983) and two-dimensional helical wheel diagrams (Schiffer & Edmundson, 1967) show the mastoparans to be distinctly amphiphilic. Circular dichroism data corroborate the theoretical predictions for the mastoparans in aqueous solution and dissolved in water/organic solvent mixtures. Fluorescence spectra and acrylamide quenching show that the tryptophan residue of the mastoparans forms part of the peptide–protein interface. Moreover, the peptide becomes distinctly helical when bound to calmodulin as shown by difference CD spectroscopy. These results are of value for the design of peptides with high affinity for calmodulin and for further study of the peptide–protein interface.

Materials and Methods

Bovine brain calmodulin was purified according to Hart et al. (1983). The affinity resin was the kind gift of Dr. Russell Hart of the University of Georgia. The concentration of calmodulin was determined from its absorbance at 276 nm by using a molar extinction coefficient of 3300 M^{-1} cm^{-1} (Klee & Vanaman, 1982). Mastoparan, mastoparan X, and *Polistes* mastoparan (salt free) were obtained from Peninsula Biochemicals (Belmont, CA). Their concentrations were determined by weight measured on a Cahn 25 automatic electrobalance. Trifluoroethanol (TFE) was obtained from Alfa Products (Danvers, MA). All other chemicals were of reagent grade or better. Ultraviolet spectra were recorded on either a Cary 219 or an SLM-Aminco DW 2C spectrophotometer.

CD spectra were measured in a Jasco J-500 spectropolarimeter equipped with a DP-500N data processor. Each spectrum was the average of 8 or 16 scans, and the final spectra were corrected for specious signals generated by solvent. The photomultiplier tube voltage was below 800 V throughout all wavelength ranges scanned. Difference CD spectra were calculated (after correction of each spectrum for background signals) by digital subtraction of the appropriate spectra. Data were expressed as mean residue ellipticity, and the helical contents were estimated according to Chen & Yang (1971).

Fluorescence measurements were made on an SLM 4800 phase fluorometer. A Hewlett-Packard 9835A computer and a 7225A plotter were used for recording and plotting spectra. Tryptophan emission from the complexes of calmodulin with mastoparan X and *Polistes* mastoparan was excited at 295 nm to minimize contributions of tyrosine (in calmodulin) to the spectra. The fluorescence emission spectra of the free peptides were recorded by using both 280- and 295-nm excitation. Excitation and emission band widths were, respectively, 2 and 4 nm. The spectra were corrected for the wavelength dependence of the detector response. Acrylamide quenching data were obtained by progressive additions of 5- or 10-μL aliquots of a 5 M buffered acrylamide solution to 2.0-mL samples of the fluorescent materials. The measured fluorescence values were then corrected for dilution and for the inner filter effect at high acrylamide concentrations. For anisotropy measurements, the exciting light (at 300 nm) was vertically polarized and passed through a slit with 1-nm band-pass. The SLM was operated in the "L" format. Emitted light was selected by use of a low intrinsic fluorescence Schott WG 345 filter which passes both horizontally and vertically polarized light equally. Thus, anisotropies could be calculated directly from the equation $r_{ss} = (I_v - I_H)/(I_v + 2I_H)$ without having to use a "G" correction term [see Lakowicz (1983)]. Fluorescence lifetimes (τ) were measured by the phase/modulation method of Spencer & Weber (1969). Modulation frequencies of 18 and 30 MHz were employed, and *p*-terphenyl (τ = 0.93 ns) was used as a fluorescence lifetime reference solution to minimize wavelength-dependent and targeting artifacts in the value of τ (Lakowicz & Cherek, 1981a; Lakowicz et al., 1981).

[1] Abbreviations: CD, circular dichroism; TFE, trifluoroethanol; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

Table I: Helical Hydrophobic Moments of Mastoparan, Mastoparan X, and *Polistes* Mastoparan and Induction of Helicity in the Peptides by Solvent Perturbation and Calmodulin Binding

peptide	$\langle\mu_H\rangle_{3-13}$[a]	$\langle H\rangle_{3-13}$[b]	$\langle\mu_\alpha\rangle$[c]	$\langle H\rangle_{av}$[d]	helicity in water[e] (%)	helicity in 70% TFE[e] (%)	helicity induced by calmodulin[f] (%)
mastoparan	0.58	0.23	0.46	0.30	15	55	
mastoparan X	0.56	0.16	0.45	0.24	7	56	61
Polistes mastoparan	0.70	−0.07	0.62	0.03	6	72	51

[a] Hydrophobic moment calculated for the 11-residue segment stretching from residue 3 to 13. [b] Average hydrophobicity for residues 3–13. [c] Mean α moment obtained by averaging the hydrophobic moments for the 4 11-residue segments derived from each 14-residue-long peptide. [d] Mean hydrophobicity obtained by averaging the hydrophobicities for the 4 11-residue segments. [e] Calculated from circular dichroism spectra (Figure 2A,B) according to Chen & Yang (1971). [f] Calculated from circular dichroism spectra (see text and Figure 2C) according to Chen & Yang (1971).

All CD and fluorescence experiments were conducted at 25 °C and pH 7.0 ± 0.05, unless otherwise mentioned. The protein–peptide complexes were studied in 20 mM *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (Tes) buffer containing 0.1 M NaCl and 1.2 mM $CaCl_2$.

RESULTS

Two-dimensional helical wheel diagrams of the three mastoparans we studied are presented in Figure 1, and simple examination is sufficient to see that these peptides are amphiphilic. Eisenberg et al. (1982) have provided a most useful quantitative analysis of information that is inherent in the two-dimensional helical wheel diagram. This is accomplished by defining the effective hydrophobicity of each amino acid side chain in terms of a hydrophobic moment vector, $\boldsymbol{H}_i$. The direction of this vector is determined by the orientation of the side chain to the helix axis. A mean helical hydrophobic moment is then defined as the mean vector μ_H, so that

$$\langle\mu_H\rangle = |\sum_{i=1}^{N} \bar{\boldsymbol{H}}_i|/N$$

where N is the number of residues. Eisenberg et al. (1982) found that in graphs of $\langle\mu_H\rangle$ vs. $\langle H\rangle$ surface-active peptides tended to cluster into a particular region of the plot. The hydrophobic moments calculated for the mastoparans are given in Table I. They are in general high, and $\langle\mu_H\rangle$ for *Polistes* mastoparan is substantially higher than those of all of the 64 α-helical segments from 26 proteins listed by Eisenberg et al. (1982). A comparison of the values for $\langle\mu_H\rangle$ and $\langle H\rangle$ shows that the peptides fall into the category of surface-seeking helices as defined by Eisenberg et al. (1982) from a plot of $\langle\mu_H\rangle$ vs. $\langle H\rangle$ for all the peptides they studied. We may conclude, therefore, that the mastoparans have high helix-forming potential and note that the tryptophan residue in mastoparan X and *Polistes* mastoparan is on the apolar side of the putative amphiphilic helix.

Circular Dichroism Measurements. Analysis of CD spectra of the three mastoparans in aqueous solution (Figure 2A) indicated 6–15% α-helical and 70–80% randomly coiled configurations (Table I). Helix could not be induced in any of the peptides by the addition of 0.085 M phosphate at pH 7.0. No appreciable change in the structure of mastoparan X was detected by CD upon increasing the phosphate concentration to 0.5 M at pH 7 [cf. Drake & Hider (1979)]. The lack of effect of 0.1 M phosphate on the CD spectrum of mastoparan, per se, was also noted by Higashijima et al. (1983). The shapes of the CD spectra changed significantly toward a more α-helical form in a medium containing 70% TFE and 30% water (Figure 2B). The contributions of α-helical structures toward these spectra were calculated to be in the range of 55–72% for the three mastoparans (Table I). This finding is in agreement with the CD data of mastoparan dissolved in neat methanol (Higashijima et al., 1983).

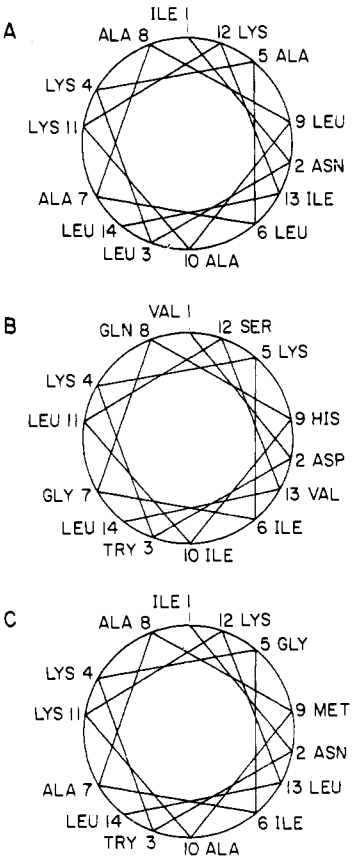

FIGURE 1: Two-dimensional helical wheel diagrams for the sequences of (A) mastoparan, (B) *Polistes* mastoparan, and (c) mastoparan X. These were drawn by using the method of Schiffer & Edmundson (1967).

In the presence of Ca^{2+}, CD difference spectra between mixtures of the peptide with calmodulin and calmodulin alone showed induced negative ellipticity with bands around 222 and 208 nm. Examples are shown in Figure 2C for mastoparan X and *Polistes* mastoparan. Analysis of these spectra suggests induced helicity in the peptides amounting to 51% and 61%, respectively, for *Polistes* mastoparan and mastoparan X (Table I) upon interaction with calmodulin. Under the same solution conditions, melittin also shows helix but becomes distinctly helical and tetrameric if exposed to high salt (especially phosphate) concentrations (Drake & Hider, 1979; Faucon et al., 1979; Talbot et al., 1979; Brown et al., 1980). Melittin becomes ~75% helical in 70% TFE but remains monomeric (F. G. Prendergast and J. Fitton, unpublished results). Maulet & Cox (1983) have shown that melittin becomes helical upon binding to calmodulin. This establishes a pattern for the behavior of at least four amphiphilic peptides interacting with calmodulin.

Table II: Anisotropies, Lifetimes, and Acrylamide Quenching Constants for Tryptophan Fluorescence of Free Mastoparan X, *Polistes* Mastoparan, Melittin, and the Complexes of These Peptides with Calmodulin

sample	r^a	$\tau_\phi{}^b$	$\tau_m{}^b$	$\langle\tau\rangle^c$	$K_{SV}{}^d$ (M^{-1})	$k_q \times 10^{-9}$ (M^{-1} s^{-1})
mastoparan X	0.032	2.5	3.5	3.0	11.7	3.9
Polistes mastoparan	0.021	3.1	3.5	3.3	11.5	3.5
melittin	0.029	3.1	3.7	3.4	17.5	5.2
mastoparan X–calmodulin	0.098	2.4	3.2	2.8	1.1	0.39
Polistes mastoparan–calmodulin	0.107	2.2	3.5	2.8	1.7	0.60
melittin–calmodulin	0.088	2.9	4.5	3.7	4.3	1.2

[a] r represents anisotropy values obtained at 25 °C in aqueous Ca^{2+}-containing buffer of pH 7 for an excitation wavelength of 300 nm and an interference filter of 5 nm band-pass centered at 345 nm placed in the emission path. [b] τ_ϕ and τ_m refer to fluorescence lifetimes measured by phase and relative demodulation, respectively, using a modulation frequency of 30 MHz. The disparity in the values of τ_ϕ and τ_m is due to heterogeneity [see Lakowicz (1983)]. [c] $\langle\tau\rangle$ is defined as $(\tau_\phi + \tau_m)/2$ and was used for calculating the bimolecular quenching constant, k_q. [d] K_{SV} is obtained from the Stern–Volmer relation $F_0/F = 1 + K_{SV}[Q]$ where F_0 is the initial fluorescence intensity and F the fluorescence intensity at a concentration of quencher [Q]. We assumed quenching to be collisional only.

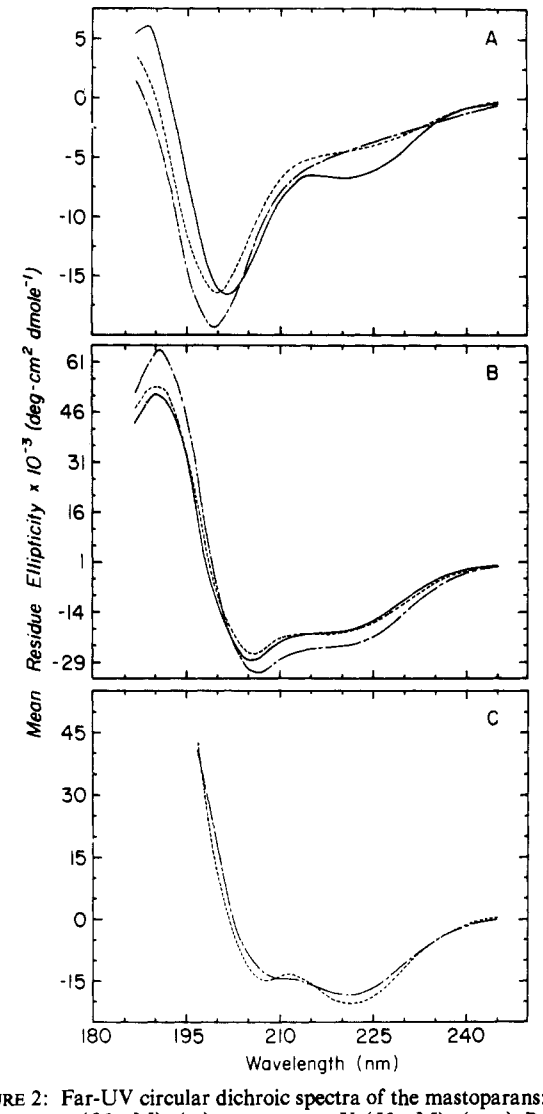

FIGURE 2: Far-UV circular dichroic spectra of the mastoparans: (—) mastoparan (56 μM); (···) mastoparan X (53 μM); (–·–) *Polistes* mastoparan (51 μM). (A) Solvent, water; (B) solvent, 70% trifluoroethanol + 30% water; (C) difference spectra between calmodulin–mastoparan X complex and calmodulin (···) and between calmodulin–*Polistes* mastoparan complex and calmodulin (–·–). Concentrations of calmodulin and the peptides were 55 μM each. Buffer contained 20 mM Tes, 100 mM NaCl, and 1.2 mM $CaCl_2$ at pH 7.0 (25 °C).

Fluorescence Measurements. Malencik & Anderson (1983b, 1984) used measurements of tryptophan fluorescence spectra and anisotropy to show that mastoparan X and *Polistes* mastoparan both interact with calmodulin. Our spectral data (not shown) are identical with theirs and show a large blue shift (22–24 nm) in the tryptophan emission from these peptides upon interaction with calmodulin. The anisotropy values for mastoparan X, *Polistes* mastoparan, and melittin are all increased upon binding of the peptides to calmodulin (see Table II), in agreement with the data of Malencik & Anderson (1983b). Our values for camodulin-bound mastoparan X and *Polistes* mastoparan are, however, 15% and 23% lower than theirs, respectively. The fluorescence anisotropy of melittin in the complex with calmodulin is 12% less than that of mastoparan X bound to this protein under the same experimental conditions. This finding, when taken with the smaller shift in emission spectra, suggests a less direct involvement of W19 of melittin in the interaction site of calmodulin than for W3 of mastoparan X and *Polistes* mastoparan. Further evidence in support of this assertion was obtained from measurements of acrylamide quenching of tryptophan fluorescence in the peptide–calmodulin complexes. Although the Stern–Volmer plots of acrylamide quenching (Eftink & Ghiron, 1976a,b) for both the free and the protein-bound peptides are linear (Figure 3), binding to calmodulin greatly reduces the bimolecular quenching constants, k_q (Table II). The magnitudes of this reduction are 10-, 6-, and 4-fold, respectively, for mastoparan X, *Polistes* mastoparan, and melittin. Our values for free melittin and calmodulin-bound melittin are comparable to those reported by Georghiou et al. (1982) for this peptide in 0.15 M NaCl and in association with egg phosphatidylcholine. The data in Table II also show that the lifetimes of tryptophan fluorescence are heterogeneous and do not change markedly when the peptides bind to calmodulin despite the very distinct changes in fluorescence intensity and anisotropy. The heterogeneity in τ for the free peptides is probably due to the existence of rotamers (Cockle & Szabo, 1981) but in the protein may be attributable to either heterogeneity or dipolar relaxation processes (Lakowicz & Cherek, 1980, 1981b). However, the marginal change in the apparent τ is particularly interesting in view of the marked spectral shift and increase in anisotropy which typically presage changes in τ. This finding underscores the need for measuring the quantum yields for calculation of the radiative lifetimes which seem likely to provide the most appropriate values for comparison of tryptophan emission from different environments (Szabo et al., 1983).

DISCUSSION

Amphiphilicity and cationic character are the features common to the mastoparans and melittin. The hydrophobic moments of residues 3–14 of the mastoparans are strikingly high compared to values for other amphiphilic peptides and puts them in the rank of surface-interacting peptides as defined by Eisenberg and co-workers (Eisenberg et al., 1982). The

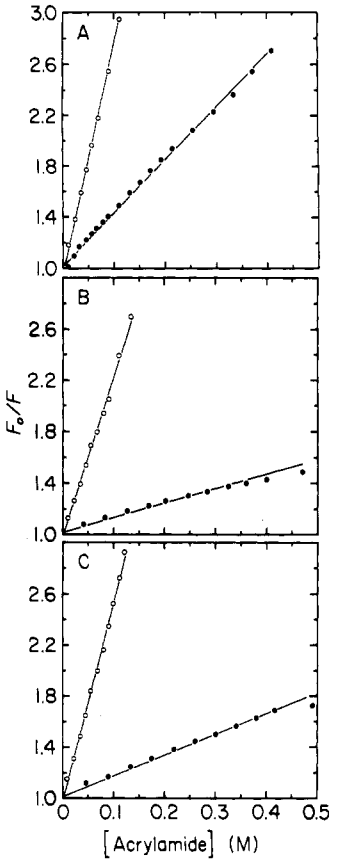

FIGURE 3: Effect of calmodulin binding on acrylamide quenching of tryptophan fluorescence emission from (A) melittin, (B) *Polistes* mastoparan, and (C) mastoparan X: (○) free peptide; (●) bound peptide. Excitation wavelength was 295 nm with a band-pass of 2 nm. Emission was observed through a monochromator set at 348 and 335 nm, respectively, for the free and bound peptides in experiments with *Polistes* mastoparan and mastoparan X, and at 350 nm for both free and bound melittin. The emission bandwidth was 4 nm. Concentrations of mastopran X and *Polistes* mastoparan (both free and bound) were 10 μM each, and an equimolar concentration of calmodulin was used. Data for the melittin experiments were obtained by using 4 μM melittin and 4 μM melittin reacted with 5 μM calmodulin. Buffer contained 20 mM Tes, 100 mM NaCl, and 1.2 mM $CaCl_2$ at pH 7.0 (25 °C).

largely random conformation of these and other amphiphilic peptides such as melittin in aqueous solution at low ionic strength (<0.15) but helical conformation in organic solvent/water mixtures suggests that the hydrophobic moment may be mostly a predictor of the conformation of such peptides given appropriate solvent conditions or a surface to which the peptide can adsorb. It probably is of little consequence what the surface is, provided that steric and polarity demands are met. Hence, binding to lipid surfaces and to proteins is not surprising. The data we have presented on peptide binding to calmodulin corroborate and extend those of other authors. The fluorescence data all support the primary involvement of the tryptophan at position 3 of mastoparan X and *Polistes* mastoparan at the interaction surface. The CD data show clearly the induction of helicity in the peptides to a similar extent as that caused by 70% TFE. We therefore infer that calmodulin provides a complementary surface for adsorption of the apolar face of the amphiphilic helix of the mastoparans and very likely to melittin. The role of amphiphilicity is obviously not absolute since the affinity constants for the binding of different peptides to calmodulin bear no predictable relation to the calculated hydrophobic moment or the apparent maximal helicity. This latter inference must be tempered by the uncertainty in the measured affinity constants. These have been measured indirectly by determining the ability of the peptides to displace calmodulin-bound enzymes of lower affinity (Malencik & Anderson, 1983b, 1984). The errors in such measurements are probably sufficient to explain the differences in the estimated K_d values for mastoparan, mastoparan X, *Polistes* mastoparan, and melittin to calmodulin [see Comte et al. (1983) and Malencik & Anderson (1983b)]. We would therefore predict that the driving force for interaction would be the entropic advantage gained from desolvation of both the Ca^{2+}-induced exposed hydrophobic site on calmodulin (LaPorte et al., 1980) and the apolar surface of the amphiphilic peptide despite the loss of configurational entropy in the peptide. This conclusion differs from that of Malencik & Anderson (1984), who inferred demand for β-structure in the peptide ligand. We do not yet know how much of the helix "surface" is required for actual interaction, but tight binding (nanomolar K_d) is apparently assured by only 2–4 turns of helix (from the estimated helicity of the calmodulin-bound mastoparans and the hydrophobic index of residues 3–14). If indeed melittin binds more tightly to calmodulin than do the mastoparans (Malencik & Anderson, 1984), this might be attributable either to enhanced affinity due to the longer helix [despite the fracture in the melittin helix; see Terwilliger & Eisenberg (1982)] or to stronger electrostatic contributions from the highly cationic carboxy-terminal hexapeptide of melittin (Habermann & Jentsch, 1967). If the extent of the binding surface can be identified, one should be able to calculate the free energy of interaction and compare this to that calculated from the K_d. The predictive value of these inferences for the design of inhibitory peptides with even greater affinities than those so far measured is apparent.

Finally, mastoparan X and *Polistes* mastoparan offer another advantage because of the sensitivity of the tryptophan fluorescence to interaction. Not only is the change in fluorescence useful for measurement of the binding affinities but it also offers a superb model for the study of the structural determinants of tryptophan fluorescence, once the binding site on the protein has been identified with the peptide docked. For example, despite the similarity in the fluorescence spectra and location on the apolar surface of the amphiphilic helix of the complexes of mastoparan X and *Polistes* mastoparan with calmodulin, there are small but significant differences in the acrylamide quenching constants and anisotropies for the single tryptophan residues in the calmodulin-bound peptides. The tryptophan in the mastoparan X–calmodulin complex is less accessible to acrylamide than that in the *Polistes* mastoparan–calmodulin complex. However, the lower anisotropy of the former suggests a higher "local" mobility of the tryptophan moiety (i.e., mobility independent of whole protein flexing or rotation) in the protein-bound mastoparan X. It is likely that this property can be varied by changing the location of the tryptophan residue on the (putative) binding surface to provide excellent models for study of intraprotein indole side chain mobilities and subsequent correlation with structural determinants of such mobility.

ADDED IN PROOF

While this work was being prepared for publication, two papers have come to our notice in which conclusions have been drawn that are identical with the ones we reached. These are by Cox et al. (1985) and Cox (1984).

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Registry No. Mastoparan, 72093-21-1; mastoparan X, 72093-22-2; *Polistes* mastoparan, 74129-19-4; melittin, 37231-28-0; tryptophan, 73-22-3.

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