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HIGH AFFINITY BINDING OF THE MASTOPARANS BY CALMODULIN

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Calmodulin exhibits high affinity, calcium-dependent binding of the mastoparans —— a group of cytoactive tetradecapeptides. The dissociation constants for the peptide-calmodulin complexes determined in 0.20 N KCl, 1.0 mM $CaCl_2$, pH 7.3 are ~0.3 nM for mastoparan, ~0.9 nM for mastoparan X, and ~3.5 nM for Polistes mastoparan. The dissociation constant for the mastoparan-calmodulin complex is the smallest known for any calmodulin binding protein or peptide, suggesting that some type of peptide-calmodulin interaction could be physiologically significant.

Calmodulin (1) and the cAMP-dependent protein kinase (2) are examples of proteins whose biological function is recognition and regulation of other protein molecules. Experiments with model peptides, often providing information on the specificity of protein-protein interactions, have helped to establish a class of recognition sequences surrounding the serine and threonine residues which can be phosphorylated by the protein kinase (3). Recent studies have shown the calcium-dependent binding of a number of small peptides by calmodulin (4,5,6,7,8). Since these peptides compete in binding, both with each other (5,6) and with enzymes such as the cyclic nucleotide phosphodiesterase (4) or myosin light chain kinase (9), they can be regarded as models for the calmodulin binding sites in calmodulin-dependent enzymes. Malencik and Anderson (5,6,9,10) noted that the peptides which calmodulin binds well (K_d < 10 μM) contain homologies consisting of a strongly basic tripeptide sequence three positions away from a pair of bulky hydrophobic residues, following a pattern which resembles the recognition sequences for the protein kinase.

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This report describes the discovery of exceptionally high affinity calmodulin binding by a group of cytoactive tetradecapeptides, the mastoparans. The mastoparans, isolated from the venoms of wasps and hornets (11,12), are promising both as model peptides and as potential calmodulin antagonists.

MATERIALS AND METHODS

Porcine calmodulin was prepared by the procedure of Schreiber et al (13); turkey gizzard myosin light chain kinase, according to Adelstein and Klee (14); and rabbit skeletal muscle troponin I, by the method of Kerrick et al (15). Dansylcalmodulin was previously prepared by us (5). The peptides were purchased from Peninsula Labs, dissolved in buffer to give solutions containing 0.02-0.1 mg/ml, and stored at -70° between use. 9-Anthroylcholine was obtained from Molecular Probes, Inc. Our buffers usually contained 45 mM MOPS, 0.2 N KCl, and 1.0 mM $CaCl_2$ dissolved in glass distilled H_2O (pH 7.3).

Fluorescence spectra and intensities were recorded using the Hitachi-Perkin Elmer MPF-2A fluorescence spectrophotometer. The spectra were corrected for the wavelength dependence of the grating transmission and detector response. Small corrections, generally less than 5% of the total intensity, were made for the protein and solvent blanks. Fluorescence anistropy measurements were obtained with the SLM 4000 fluorescence polarization spectrophotometer.

RESULTS

The presence of tryptophan in two of the mastoparans, mastoparan X from the Japanese hornet and Polistes mastoparan (Table I), facilitates fluorescence binding measurements. We previously used the changes in the intrinsic fluorescence spectra of tryptophan containing peptides, ACTH and glucagon, to follow their association with calmodulin (5,6). The fluorescence anisotropy $((I_{||} - I_{\perp})/(I_{||} + 2I_{\perp}))$ is also useful since the

Table I. Sequences of the Mastoparans[1]

Mastoparan	Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2
Mastoparan X	Ile-Asp-Trp-Lys-Gly-Ile-Ala-Ala-Met-Ala-Lys-Lys-Leu-Leu-NH_2
Polistes Mastoparan	Val-Asp-Trp-Lys-Lys-Ile-Gly-Gln-His-Ile-Leu-Ser-Val-Leu-NH_2

[1] from references 11,12

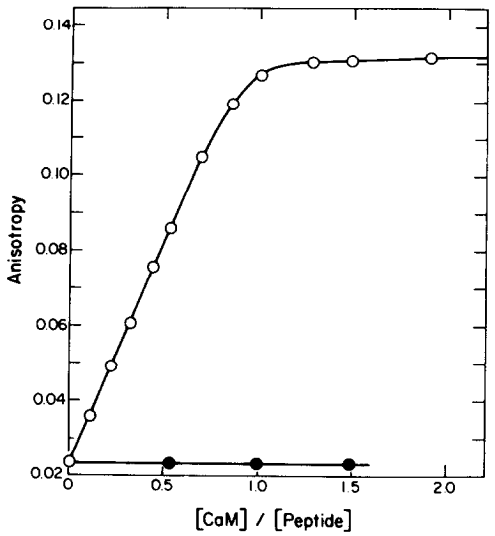

Figure 1. Stoichiometric titration of Polistes mastoparan with calmodulin. The fluorescence anisotropies were measured using an excitation wavelength of 294 nm, with a bandpass of 2 nm. The emitted light passed through Corning glass CS 0-54 filters. Conditions: 5.0 μM Polistes mastoparan in 0.20 N KCl, 5.0 mM MOPS, pH 7.3 (25.0°).
○ 1.0 mM $CaCl_2$ added ● 0.1 mM EDTA added

association of such small peptides with proteins as large as calmodulin greatly reduces their rates of rotational diffusion (16). The direct contribution of calmodulin to the background flurorscence intensity is minimized by excitation at 290-295 nm, where the tyrosine residues of calmodulin absorb only slightly (5,6).

Figure 1 shows the stoichometric increase in fluorescence anistropy occurring when Polistes mastoparan is titrated with calmodulin in the presence of Ca^{++}. Saturation is reached on the addition of one mol calmodulin per mol peptide, indicating that calmodulin contains one high affinity binding site for Polistes mastoparan. Titrations of both Polistes mastoparan and mastoparan X in solutions containing 10^{-4} M EDTA showed no interaction with calmodulin. Intensity measurements on the fluorescent conjugate dansylcalmodulin revealed a weak association at higher concentrations of mastoparan, suggesting a K_d of 10^{-4} M or larger when EDTA is present. The fluorescence spectra of Polistes mastoparan and mastoparan X undergo large shifts on complex formation (Figure 2). The wavelength of the emission

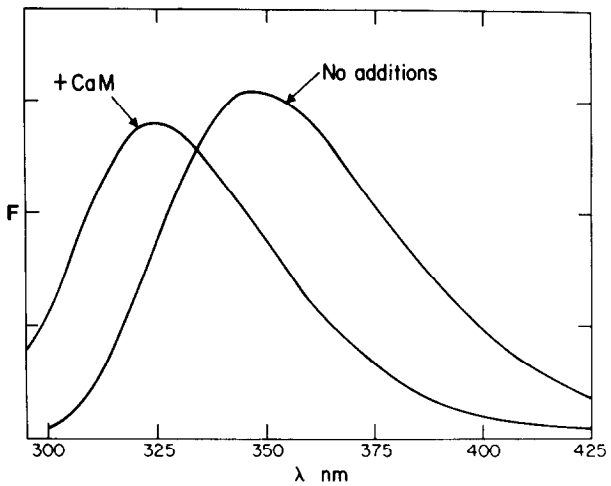

Figure 2. Effect of calmodulin binding on the fluorescence spectrum of Polistes mastoparan. Calmodulin (10.0 μM) was added to Polistes mastoparan (8.0 μM) in a solution containing 0.20 N KCl, 5.0 mM MOPS, 1.0 mM $CaCl_2$, pH 7.3 (25.0°). Excitation: 290 nM. The band widths of excitation and emission were 3 and 5 nm, respectively.

maximum decreases from 348 nm to 325 nm in both cases, demonstrating that the local environment of the tryptophan residues in the bound peptides is comparable to the protected environments found in many protein molecules.

Smooth muscle myosin light chain kinase, an absolutely calmodulin-dependent enzyme, contains a binding site for the fluorescent probe 9-anthroylcholine which may correspons to the ATP binding site (9). Interaction of the enzyme with calmodulin increases its affinity for 9-anthroylcholine, with the dissociation constant decreasing from 20 μM to 6.4 μM. The resulting changes in the fluorescence of the probe are sensitive to calmodulin binding by the enzyme and are useful in evalution of the competition with high affinity calmodulin binding proteins such as troponin I (TnI). The extent of dissociation of the enzyme-calmodulin complex can be determined from the fractional change (ΔF) in the fluorescence intensities (F).

$$\Delta F = \frac{F_{MLCK \cdot CaM} - F_{obs}}{F_{MLCK \cdot CaM} - F_{MLCK}}$$

Figure 3 shows the fractional changes in the fluorescence of 9-anthroycholine when solutions containing the myosin light chain kinase-calmodulin complex (0.5 μM) are titrated with the three mastoparans and with TnI - the most widely

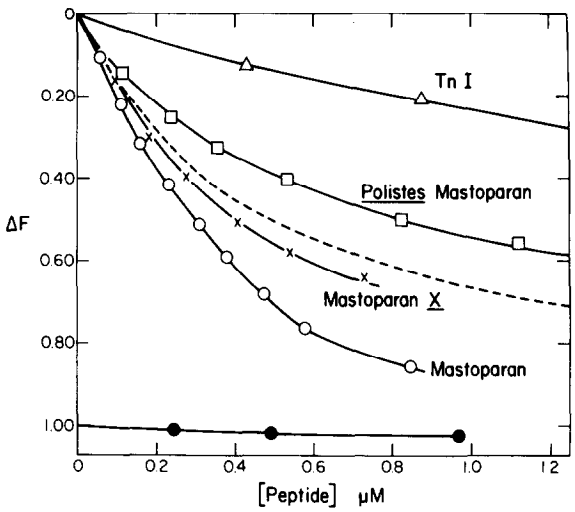

Figure 3. Dissociation of the smooth muscle myosin light chain kinase-calmodulin complex by the mastoparans. A 0.50 μM solution of the enzyme-calmodulin complex was titrated with mastoparan (O), mastoparan X (X), Polistes mastoparan (□), and with rabbit muscle troponin I (Δ). The dashed line shows the displacement expected when the enzyme and the competing peptide bind calmodulin equally well. A control experiment showing the effect of mastoparan on the enzyme alone is also shown (●). Conditions: 5.0 μM 9-anthroylcholine, 0.20 N KCl, 45 mM MOPS, 1.0 mM $CaCl_2$, 1.0 mM dithiothreitol, pH 7.3 (25.0°). Excitation: 360 nm. Emission: 460 nm.

used model calmodulin-binding protein (17). The mastoparans proved surprisingly efficient in competition, with the affinities of mastoparan and of mastoparan X actually surpassing the affinity of the enzyme for calmodulin. (The dashed line in Fig. 3 illustrates the dissociation expected when the enzyme and the competing peptide bind calmodulin equally well.) The addition of mastoparan to the enzyme-9-anthroylcholine complex alone has negligible effects on the fluorescence.

The relative values of the dissociation constants for the two competing equilibria can be evaluted even when the individual values are beyond the range of direct determination.

$$\text{MLCK}\cdot\text{CaM} \underset{K_{\text{MLCK}}}{\rightleftharpoons} \text{MLCK} + \text{CaM}$$

$$\text{P}\cdot\text{CaM} \underset{K_{\text{P}}}{\rightleftharpoons} \text{P} + \text{CaM}$$

We showed that $\frac{K_{\text{MLCK}}}{K_p} = \frac{\Delta F^2}{(1-\Delta F)} \frac{[\text{MLCK}]_o}{([\text{P}]_o - \Delta F[\text{MLCK}]_o)}$ where $[\text{MLCK}]_o$ and $[\text{P}]_o$

represent the total concentrations of the enzyme and the competing peptide, respectively (6). Application of this equation to the data in Figure 3 gives the following values for the ratio K_{MLCK}/K_p: TnI, 0.035 ± 0.01 (data at higher concentrations than those shown were included); Polistes mastoparan, 0.40 ± 0.02; mastoparan X, 1.5 ± 0.2; and mastoparan, 4.9 ± 0.3. The value for K_{MLCK} in the presence of 9-anthroycholine has been found to be ~1.4×10^{-9} M (6) while that for K_{TnI} has been independently determined to be ~4×10^{-8} M (18,19). Taking these values into account, we calculate the following dissociation constants for the peptide-calmodulin complexes: mastoparan, ~3×10^{-10} M; mastoparan X, ~9×10^{-10} M; and Polistes mastoparan, ~3.5×10^{-9} M.

DISCUSSION

Mastoparan, a tetradecapeptide isolated from the venom of the Vespid wasp, has a higher affinity for calmodulin that any ever found - either in a calmodulin-dependent enzyme or in another model peptide. Enzymes regulated by calmodulin typically have K_m's in the range of 1-2 nM. The two most effective known calmodulin binding peptides other than the mastoparans are the vasoactive intestinal peptide (VIP), with 28 amino acid residues and K_d ~50 nM (6), and melittin, with 26 residues and K_d ~3 nM (8).

The sequence of mastoparan (Table I) conforms to the ideal model or recognition sequence envisioned by us from observation on other small peptides (refer to the introduction). The large proportion of bulky, hydrophobic side chains and a high predicted α-helix potential distinguish mastoparan from all the other known calmodulin binding peptides. The structure prediction rules of Chou and Fasman (20) give overall values of $\langle P_\alpha \rangle = 1.22$ and of $\langle P_\beta \rangle = 1.06$, indicating that the α-helix and the β-strand are both possible secondary structures with the former preferred. The α-helical nucleus at positions 5-10 corresponds to $\langle P_\alpha \rangle = 1.41$. Since most of the other model peptides gave higher values for $\langle P_\beta \rangle$ than for $\langle P_\alpha \rangle$, we suggested that β-sheet formation is involved in peptide binding by calmodulin (6). If the highly efficient binding of mastoparan reflects assumption of the

most stable conformation, then either the other peptides are also bound in the α-helical conformation or calmodulin recognizes both α-helices and β-strands.

The exceptional affinity of mastoparan for calmodulin suggests that it could be a valuable antagonist for use in in vitro studies of calmodulin-dependent processes. Whether the interaction between calmodulin and mastoparan occurs in vivo is unknown. Mastoparan is known to stimulate degranulation and release of histamine from mast cells (11). However, the tenacity of binding and strong calcium dependence found in such a small peptide suggest that some type of peptide-calmodulin interaction could be physiologically significant. A general precedent for regulation by intracellular peptides exists in the 8000 dalton heat stable inhibitor of the cAMP-dependent protein kinase (21).

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