Calmodulin and Troponin C: A Comparative Study of the Interaction of Mastoparan and Troponin I Inhibitory Peptide [104–115]†

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ABSTRACT: Recent studies using bee and wasp venom peptides have led to the hypothesis that proper complex formation with calmodulin (CaM) requires the presence of a basic amphiphilic helix on the surface of the target protein [Cox, J. A. (1984) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 43*, 3000]. We have tested this hypothesis by examining CaM and troponin C (TnC) complex formation with two basic peptides, the wasp venom tetradecapeptide mastoparan and the physiologically relevant synthetic troponin I (TnI) inhibitory peptide [104–115], using far-ultraviolet circular dichroism as a secondary structure probe. Complex formation between mastoparan and either CaM or TnC results in an increase in helical content, whereas the helical content of TnI inhibitory peptide does not increase when bound to either protein. Significantly, mastoparan is 78% α-helical in a 50% solution of the helix-inducing solvent trifluoroethanol and has a high helix-forming potential according to the Chou–Fasman rules while TnI inhibitory peptide contains none and is not predicted to have any. We interpret these data as indicating that these peptides exhibit substantially different secondary structures upon binding to CaM or TnC. The ability of mastoparan to regulate the acto–subfragment 1–tropomyosin ATPase has also been examined. Mastoparan and TnI inhibitory peptide inhibited 31% and 45% of the activity, respectively. TnC and CaM promote differing degrees of Ca^{2+}-sensitive release of inhibition by both peptides. Sequence comparison suggests that the basic residues present in both peptides are important for binding. However, we conclude that an α-helical structure is not a prerequisite for the binding of target proteins to CaM and TnC.

The Ca^{2+} binding regulatory proteins calmodulin (CaM)[1] and troponin C (TnC) are remarkably similar in many respects. Their amino acid sequences show approximately 50% direct homology and 70% conservative homology (Dedman et al., 1978; Watterson et al., 1980). This high level of sequence homology is reflected in the tertiary structures of the molecules (Herzberg & James, 1985; Babu et al., 1985). Both TnC and CaM contain two major folding domains separated by a long central helix. Each major domain contains two Ca^{2+} binding sites based on the EF had helix–loop–helix structural motif first discovered in carp parvalbumin (Kretsinger & Nockolds, 1973; Collins et al., 1973; Vanaman et al., 1977). TnC has two classes of Ca^{2+} binding sites. The higher affinity Ca^{2+}–Mg^{2+} sites are sites III and IV in the C-terminal half of the molecule. These sites bind Ca^{2+} or Mg^{2+} with association constants of 2×10^7 and 5×10^3 M^{-1}, respectively. Sites I and II in the N-terminal half of the molecule are the Ca^{2+}-specific sites which bind Ca^{2+} with an association constant of 2×10^5 M^{-1} (Potter & Gergely, 1975; Johnson & Potter, 1978). CaM, in contrast, is specific for Ca^{2+} and contains four sites with association constants falling within 1 order of magnitude, ranging from 10^5 to 10^6 M^{-1}. Similarly to TnC, however, these four sites can be divided into two subclasses of higher and lower affinity. NMR studies on CaM fragments suggest that as with TnC, sites III and IV in the C-terminal half of the molecule are the higher affinity sites while sites I and II are of lower affinity (Andersson et al., 1983; Aulabaugh et al., 1984; Klevit et al., 1984).

The mechanism by which Ca^{2+} causes activation of the various enzyme systems regulated by these proteins involves Ca^{2+}-induced conformational changes, which result in the presentation of binding interfaces capable of binding target proteins with high affinity (LaPorte et al., 1980). On the basis of a variety of studies, it appears that the α-helical segment on the N-terminal side of the site III Ca^{2+} binding loop, i.e., the III_N helix, is important in a number of binding interactions for both TnC and CaM [for a review, see Cachia et al. (1985)]. In particular, the data indicate that this is a site of interaction with the inhibitory peptide region of TnI, residues 104–115 of TnI, as well as a variety of peptide hormones, toxins, and phenothiazine drugs. Much recent work has focused on determining what common features enable these various peptides and drugs to bind to these proteins (Cachia et al., 1985). Of particular interest to us in this matter have been those which addressed the question of the secondary structure adopted by a peptide in order to form a binding interface (McDowell et al., 1985; Maulet & Cox, 1983; Cox et al., 1985; Puett et al., 1983; Malencik & Anderson, 1983a). Several of these studies have utilized bee or wasp venom peptides such as melittin or one of the mastoparans which are capable of forming amphiphilic α-helices upon binding to hydrophobic surfaces such as the CaM binding site (Maulet & Cox, 1983; Cox et al., 1985; McDowell et al., 1985).

Since so many unrelated proteins and peptides can bind to CaM or TnC with differing association constants, it is important to determine whether α-helical secondary structure is a prerequisite for binding to these regulatory proteins. To

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[1] Abbreviations: CaM, bovine brain calmodulin; TnC, rabbit skeletal troponin C; TnI, troponin I; TM, rabbit cardiac tropomyosin; S1, rabbit myosin subfragment 1; EDTA, ethylenediaminetetraacetic acid; TFE, trifluoroethanol; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; f_H, fraction helix; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate.

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investigate this matter, we have examined the secondary structures of TnC, CaM, mastoparan, synthetic TnI inhibitory peptide [104–115], and their respective complexes using far-ultraviolet circular dichroism as a probe. Furthermore, we have evaluated the abilities of mastoparan and the TnI inhibitory peptide both to inhibit the acto–S1–TM ATPase and to exhibit Ca^{2+}-dependent release of inhibition upon addition of TnC or CaM. By doing so, we address the question of whether the similarities which TnI inhibitory peptide and mastoparan apparently share in their binding interfaces enable mastoparan to also bind to F-actin–TM, regardless of significant differences in their secondary structures.

Materials and Methods

Both mastoparan and TnI inhibitory peptide [104–115] were synthesized by using the general procedures for solid-phase peptide synthesis on a Beckman Model 990 synthesizer. The crude synthetic materials were purified by high-performance liquid chromatography techniques using CM-300 weak cation-exchange resin and RP-P C18 reversed-phase resin, both supplied by SynChrom, Linden, IN.

Protein Preparation. TnC was isolated from native rabbit skeletal muscle troponin as reported by Chong and Hodges (1981). Rabbit cardiac tropomyosin (TM) was extracted and purified as described by Pato et al. (1981). G-actin was purified from rabbit skeletal muscle acetone powder as described previously (Spudich & Watts, 1971). Myosin subfragment 1 (S1) was prepared by the method of Weeds and Taylor (1975), as modified by Talbot and Hodges (1981). The S1 purification by DEAE-cellulose chromatography (Talbot & Hodges, 1981) provided two fractions: S1(A1) and S1(A2). These fractions were pooled for use in these experiments. The K^+-EDTA ATPase activity of the S1(A1,A2) preparations was 360 nmol of PO_4^{2-} min^{-1} (mg of S1)$^{-1}$.

CaM was prepared from 1 kg of bovine brain as follows, using modifications of other published procedures (Watterson et al., 1976; Gopala-Krishnan & Anderson, 1982; Brzeski et al., 1983). The brain was homogenized by blending in 2 volumes of the following buffer system: 50 mM Tris-HCl, 1 mM EDTA, 1 mM β-mercaptoethanol, and 0.5 mM phenylmethanesulfonyl fluoride at pH 7.5 for 15 s. The homogenate was centrifuged at 15000g for 30 min. The pellet and supernatant were saved, and the pellet was resuspended as before in the same volume of buffer and then recentrifuged. The two supernatants were combined and saved, and the pellet was discarded. The combined supernatants were filtered through four layers of cheesecloth to remove suspended lipids (without vacuum). The supernatant was then subjected to isoelectric precipitation at pH 4.3 by the following method: 6 M acetic acid was added dropwise with constant sitrring until the pH reached 4.3. The solution was then allowed to stand at 4 °C for 1 h, followed by centrifugation at 15000g for 30 min. The pellets were collected and resuspended in a minimum volume of the homogenization buffer, and the pH was adjusted to 7.5 with 1 M Tris base. Approximately 300–400 mL of homogenization buffer per kilogram of brain was required. This solution was subjected to heat denaturation at 100 °C for 5 min, and the denatured protein was removed by centrifugation at 15000g for 30 min after the preparation was cooled to 4 °C in ice. The supernatant was recovered and dialyzed against the following buffer: 50 mM Tris-HCl, 1 mM β-mercaptoethanol, and 0.1 mM $CaCl_2$, pH 7.5. After dialysis, the $CaCl_2$ concentration in the sample was raised to 5 mM, and the sample was loaded at room temperature onto a phenyl-Sepharose column (2.5 × 45 cm) equilibrated in the dialysis buffer. The column was washed with 10 bed volumes of dialysis buffer followed by 10 bed volumes of 50 mM Tris-HCl, 1 mM β-mercaptoethanol, 0.5 M NaCl, and 0.1 mM $CaCl_2$, pH 7.5. CaM was eluted from the column with 50 mM Tris-HCl, 1 mM β-mercaptoethanol, and 1 mM EGTA, pH 7.5. CaM fractions were dialyzed first against 1 mM EGTA and 0.1 M NaCl and finally against 0.01 M NH_4HCO_3. The CaM was lyophilized and stored at −20 °C. SDS–urea gels were used to establish that the protein was pure.

Preparation of Circular Dichroism Samples. Purified proteins or peptides were dissolved directly in a sample buffer consisting of 20 mM NH_4HCO_3, 50 mM KCl, and 1 mM EGTA, pH 7.3. The pH of the sample was determined and adjusted to 7.3 with either 5 M HCl or 5 M KOH. Both protein and peptide samples were eventually filtered through a 0.45-μm Millipore filter to remove particulate matter before use. However, in the case of the protein samples, this filtration step was preceded by overnight dialysis vs. the sample buffer (20 mM NH_4HCO_3, 50 mM KCl, and 1 mM EGTA, pH 7.3).

Protein and Peptide Concentration. All protein concentrations were determined by amino acid analysis using a Dionex 502 amino acid analyzer, except for S1, which was determined by absorbance, $E^{1\%}_{280nm}$ = 7.9 (Talbot & Hodges, 1981).

Circular Dichroism Spectra. The CD experiments were conducted at 25 °C on a JASCO J-500c spectropolarimeter fitted with a thermostated cell holder and interfaced with a JASCO DP-500N data processor. Experiments were conducted in the following manner. In order that all the spectra taken during a titration of either CaM or TnC with the peptides would be directly comparable, all the volumes in the standard single-component spectra were adjusted to equal those of the actual interaction experiments. Data were expressed in observed ellipticities, θ_r (millidegrees). Errors in the observed values are ±0.2 mdeg. The protein and peptide concentrations used were as follows: TnC, 33.9 μM; CaM, 31.0 μM; mastoparan, 1.0 mM; TnI inhibitory peptide, 0.8 mM. The volumes of TnC and CaM solutions used were 800 μL. The number of molar equivalents of either peptide added to TnC or CaM in the experiments was always 1.0, with the exception of mastoparan addition to TnC, where a value of 1.1 was used.

Calculation of α-Helical Residues. Ellipticity values at 222 nm were converted to mean molar residue ellipticity values by using the equation:

$$[\theta]_{222} = \theta M_r / 100LC$$

where θ = degrees, L = cell path length (decimeters), C = concentration (grams per milliliter), and M_r = mean residue molecular weight calculated from the appropriate amino acid sequence(s). The fraction helix, f_H, was calculated by using the empirical equation obtained by Chang et al. (1978):

$$f_H = [\theta]_{222} / [\theta]^{\infty}_H (1 - k/n)$$

where $[\theta]^{\infty}_H$ is the mean molar residue ellipticity of an infinite helix (= −37 400°), k is the chain length dependence factor (=2.5), and n is the appropriate number of residues in a typical α-helical segment (=9). The number of α-helical residues was then calculated by multiplying f_H by the total number of residues in the protein, peptide, or complex.

ATPase Assays. ATPase assays were performed with an automatic pH-stat apparatus consisting of a Radiometer TTT-2 titrator, an SBR2c Titrigraph, and an SBU1μ syringe buret (Cotê & Smillie, 1981). Assay samples, 2 mL in volume, were placed in glass vials and stirred continuously at 25 °C. The titrant was 5–10 mM standardized KOH. The actin-activated ATPase was measured in an ATPase assay buffer

Table I: Effects of Ca^{2+} and TFE on TnC and CaM Secondary Structure

	TnC			CaM		
buffer	$[\theta]_{222}$ (deg)	HR[a]	ΔHR[b]	$[\theta]_{222}$ (deg)	HR	ΔHR
$-Ca^{2+}$	−10855	64		−14465	79	
			34			16
$+Ca^{2+}$	−16568	98		−17299	95	
			12			5
50% TFE	−18622	110		−18321	100	
$-Ca^{2+}$	−10960	64		−14161	78	
			32			9
50% TFE	−16321	96		−15878	87	
			17			15
$+Ca^{2+}$	−19250	113		−18627	102	

[a] HR is the number of helical residues. [b] ΔHR is the change in the number of helical residues.

(30 mM KCl, 0.1 mM EGTA, 5 mM $MgCl_2$, 2.5 mM Na_2-ATP, and 5 mM Tris at pH 7.8). The purified proteins for the assay were prepared as follows: TM, TnC, and CaM (~3 mg/mL) were dissolved in the Mg^{2+}-ATPase assay buffer (minus ATP plus 2 mM β-mercaptoethanol). Proteins were dialyzed against this buffer at 4 °C. Native Tn (~3 mg/mL) was dissolved in the Mg^{2+}-ATPase assay buffer (minus EGTA and ATP, plus 2 mM β-mercaptoethanol) and dialyzed against this buffer at 4 °C.

TnI is well-known for its solubility problems. In these experiments, TnI was dissolved (~3 mg/mL) and dialyzed in 8 M urea, 1 M KCl, 2 mM DTT, and 10 mM Tris-HCl buffer (pH 7.8). The samples were next dialyzed against 1 M KCl, 2 mM DTT, and 10 mM Tris-HCl buffer (pH 7.8) and then finally against 0.5 M KCl, 2 mM DTT, and 10 mM Tris-HCl buffer (pH 7.8). The dialysis of TnI was carried out under a nitrogen atmosphere to prevent oxidation of Cys residues (Horwitz et al., 1979). Control experiments indicated that the small increase in KCl concentration from the titration of TnI in 0.5 M KCl did not affect the ATPase assay (Chong et al., 1983). TnI peptide and mastoparan peptide were dissolved in ATPase buffer (minus ATP). S1 was dialyzed against the Mg^{2+}-ATPase buffer (minus ATP) for a maximum of 4 h. G-actin was polymerized by the addition of 1 M KCl to a final concentration of 30 mM. The TM to actin ratio used in these experiments was 1:7. The actin concentration was held constant at 1.5 μM while the S1 concentration was 3.0 μM. When free Ca^{2+} was required, a solution of 10 mM $CaCl_2$ in assay buffer (minus EGTA and ATP) was added to give a final free Ca^{2+} concentration of 0.2 mM.

The actin and S1 concentrations were adjusted so that the ATPase rates were in the range of 0.22–0.4 μmol of phosphate liberated/min. All results are reported in terms of percent activity of the control rate obtained from the ATPase rate of acto–S1–TM. For each individual assay, the control rate was measured (100%) and the percent change in ATPase rate produced by subsequent addition of proteins or peptides was calculated directly from the change in ATPase rate. The concentrations of TnI, TnI inhibitory peptide, and mastoparan were such that dilution of the acto–S1–TM concentration did not exceed 4%. Thus, the effects of the addition of proteins or peptides cannot be ascribed to dilution effects. Myosin S1 had a low ATPase rate of approximately 0.04 μmol of phosphate liberated/min which never exceeded more than 5% of the initial control ATPase rate. Thus, no correction was made for this activity.

Two procedures for the ATPase assays were used in this study: the continuous titration method and the multiple tube assay procedure which are described below.

Continuous Titration Method. This method is employed for the analysis of the effect which one variable has on the ATPase activity, for example, the inhibition by TnI inhibitory peptide (Figure 5). In this procedure, a single assay vial of acto–S1–TM was titrated with the variable protein or peptide (TnI, TnI, peptide, or mastoparan). The effect of accumulated variable on the ATPase activity was determined after each consecutive addition of that variable.

Multiple Tube Assay. This assay is used to determine the effect of three variables on the ATPase activity, for example, the release of inhibition due to TnI by TnC in the absence and presence of Ca^{2+} (Figure 6). This procedure consists of three measurements per assay vial. Six assay vials with identical concentrations of acto–S1–TM and 1.5 nmol of inhibitor (TnI, TnI, inhibitory peptide, or mastoparan) were prepared. The activity of each vial is measured prior to and following the addition of the protein (TnC or CaM). A third measurement is made after the addition of Ca^{2+} ion. The concentration of the protein (second component) is varied on the six assay vials, while the Ca^{2+} concentration (third component) is identical in each assay vial.

Hence, the continuous titration method is the most effective assay for examining one variable, since it monitors directly the difference in ATPase activity between the consecutive additions of the variable, while the multiple tube assay most accurately monitors the effect of the three different variables on each other.

The ATPase results are most accurate when determining measurements on the same vials and least accurate when making comparisons between different vials.

Due to day to day variations in the ATPase assays, the data for each set of experiments were collected on the same day. Replicates of each experiment were carried out on different days to ensure that the results were qualitatively similar.

Results

Binding of TnI Inhibitory Peptide to TnC and CaM. Figure 1 contains the CD spectra for TnC and TnI inhibitory peptide and illustrates the changes in ellipticity brought about by complex formation and/or Ca^{2+} binding. It is clear from the spectra that regardless of buffer conditions (±Ca^{2+}) the TnI inhibitory peptide contains no α-helical structure. Even in the presence of the strong helix-inducer trifluoroethanol (50% TFE), no α-helix is present. In contrast, the TnC spectra are typical of proteins containing significant amounts of α-helix. Furthermore, as shown in Table I, comparison of the percent helix in the plus and minus Ca^{2+} states corresponds to an increase in α-helical content from 40% ($[\theta_{222}] = -10855°$) in the absence of Ca^{2+} to 61% ($[\theta_{222}] = -16568°$) in its presence. These values are consistent with those obtained previously (Johnson & Potter, 1978). Graphical summation of the spectra of TnC and TnI inhibitory peptide yields the theoretical spectra which would occur if complex formation either did not take place or did not involve any structural change. Comparison of the observed and theoretical spectra for the complex in panels A and B Figure 1 indicates that some additional α-helix is formed as a result of complex formation. The approximate number of residues involved can be calculated as the difference between the number of residues in helical form

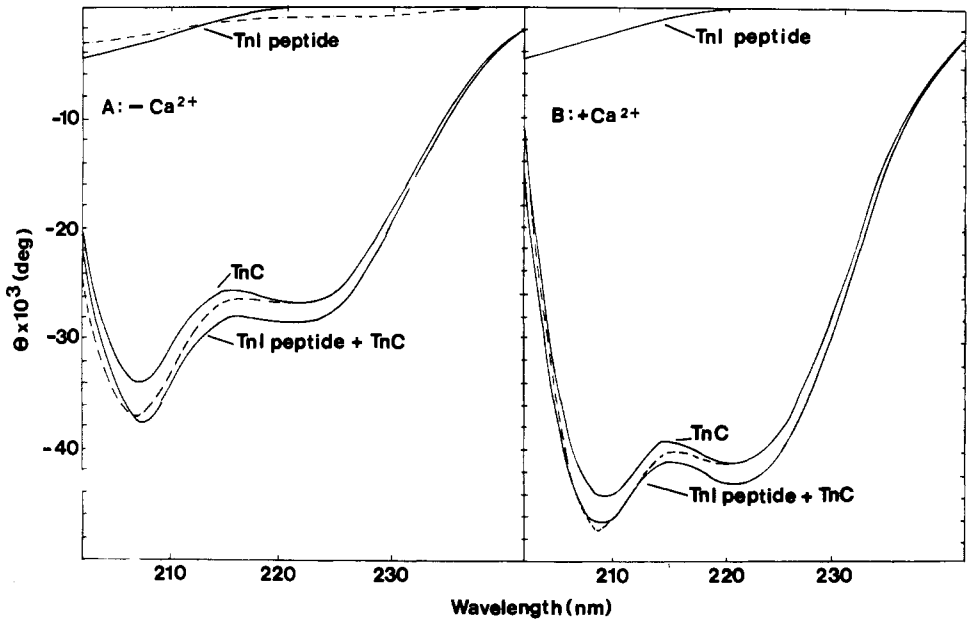

FIGURE 1: Far-ultraviolet circular dichroism spectra of TnI inhibitory peptide and TnC. (A) Spectra in the absence of Ca^{2+}; spectrum of TnI inhibitory peptide in 50% TFE (-·-). (B) Spectra in the presence of Ca^{2+} of TnI inhibitory peptide, TnC, and TnC–TnI inhibitory peptide (1:1) and theoretical circular dichroism spectrum calculated for TnI inhibitory peptide–TnC (---). Buffers used contained 50 mM KCl, 20 mM NH_4HCO_3, and 1 mM EGTA at pH 7.3 (minus Ca^{2+}) or the same plus 2.4 mM $CaCl_2$ (plus Ca^{2+}).

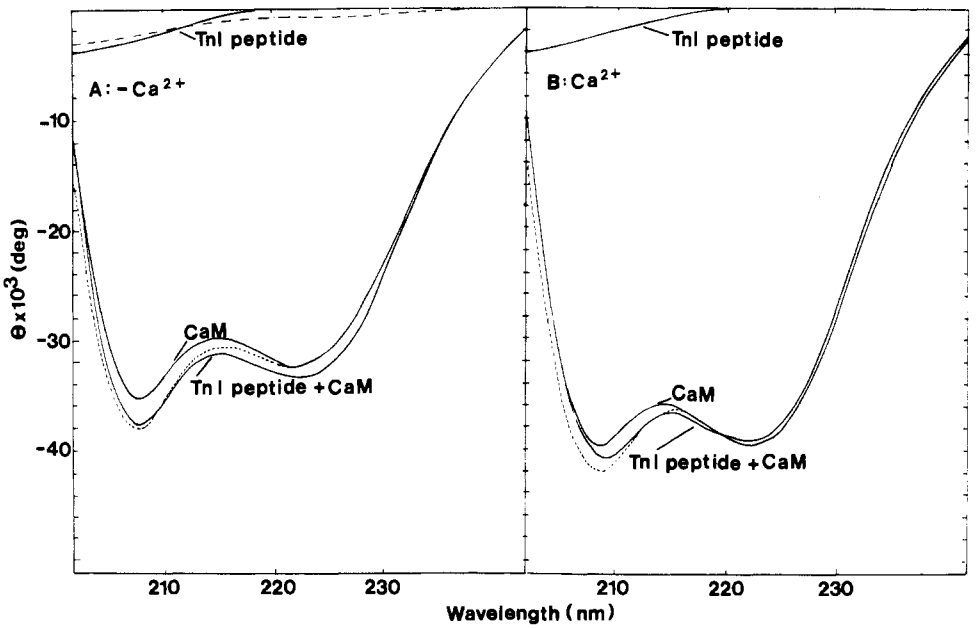

FIGURE 2: Far-ultraviolet circular dichroism spectra of TnI inhibitory peptide and CaM. (A) Spectra in the absence of Ca^{2+}; spectrum of TnI inhibitory peptide in 50% TFE (-·-). (B) Spectra in the presence of Ca^{2+} of TnI inhibitory peptide, CaM, and 1:1 mixture of peptide and CaM in 50 mM KCl, 20 mM NH_4HCO_3, and 1 mM EGTA at pH 7.3; plus Ca^{2+} buffer also contained 2.4 mM $CaCl_2$. Theoretical circular dichroism spectra calculated for TnI inhibitory peptide–CaM (---).

in the complex, f_H(complex) × residues in complex, and the number of residues in helical form in the individual components (see Materials and Methods for details). Table II shows that only three to four residues, i.e., approximately one α-helical turn, are induced upon complex formation. Since Ca^{2+} binding to TnC results in approximately 30 additional α-helical residues (Table II), it appears that, compared to the effects of Ca^{2+}, binding of the TnI peptide involves a fairly minor change in the conformation of TnC.

Figure 2 displays the CD spectra for the interaction between CaM and TnI inhibitory peptide in the presence and absence of Ca^{2+}. Comparison of the CaM spectra in panels A and B demonstrates that Ca^{2+} binding causes a significant increase in the α-helical content of CaM, corresponding to an increase in percent helix from 54% ($[\theta]_{222}$ = −14 465°) to 64% ($[\theta]_{222}$ = −17 299°). As with TnC, a small increase in the negative ellipticity at 222 nm upon TnI inhibitory peptide binding occurs both with and without Ca^{2+} present. This increase corresponds to the induction into α-helix of only 2–3 residues, far fewer than the 15–16 induced by Ca^{2+} binding (Table II).

Binding of Mastoparan to TnC and CaM. The CD spectra of TnC, mastoparan, and their complex are shown in Figure 3. In contrast to TnI inhibitory peptide, mastoparan exhibits some negative ellipticity at 222 nm; $[\theta]_{222}$ = −7547° in benign solvent. This value corresponds to an α-helical content of 25% or approximately three residues. The inherent potential for mastoparan to assume an α-helical conformation appears to be significantly greater than this would suggest, however, since

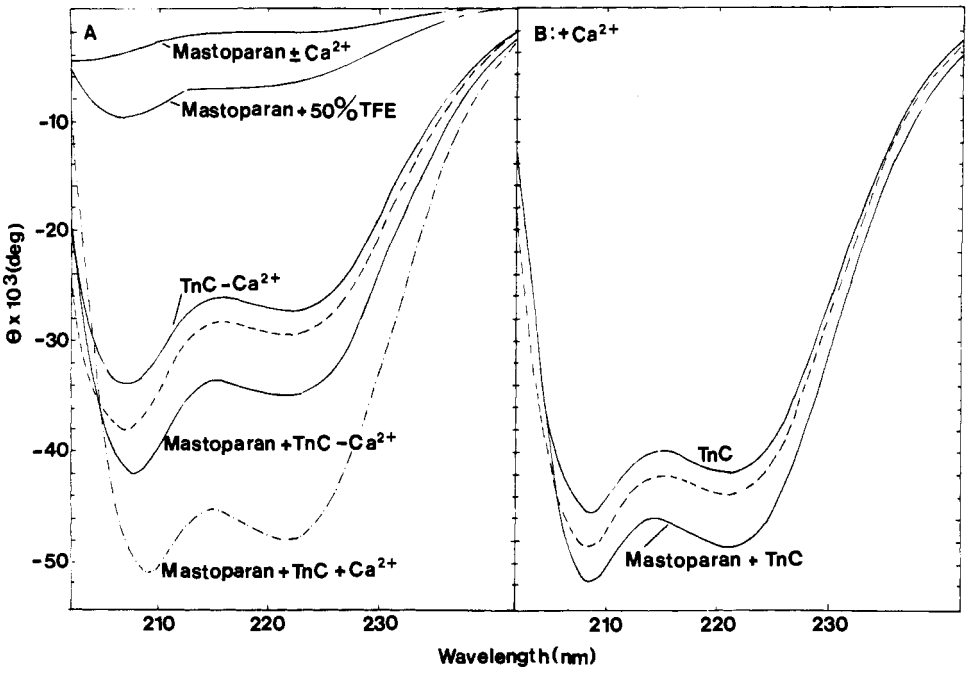

FIGURE 3: Far-ultraviolet circular dichroism spectra of mastoparan and TnC. (A) Spectra of mastoparan in 50% TFE and spectra in the absence and presence of Ca^{2+}. In the absence of Ca^{2+}, the spectra of TnC and TnC–mastoparan (1:1) and the theoretical circular dichroism spectrum calculated for TnC–mastoparan (- - -) are shown. In the presence of Ca^{2+}, the spectrum of TnC–mastoparan (–·–) is shown. (B) Spectra of TnC and TnC–mastoparan (1:1) and the theoretical spectrum of TnC–mastoparan in the presence of Ca^{2+}. Buffers used contained 50 mM KCl, 20 mM NH_4HCO_3, and 1 mM EGTA at pH 7.3 (minus Ca^{2+}) or same plus 2.4 mM $CaCl_2$ (plus Ca^{2+}).

Table II: Number of Residues Induced in an α-Helical Conformation by Mastoparan, TnI Inhibitory Peptide, and Ca^{2+} in TnC, CaM, or the Peptide–Protein Complexes

presence or absence of metal ion	peptide added	TnC–peptide	CaM–peptide
$-Ca^{2+}$	mastoparan	17	9
$+Ca^{2+}$		14	8
$-Ca^{2+}$	TnI peptide	4	3
$+Ca^{2+}$		3	2

presence or absence of peptide	metal ion added	TnC or TnC–peptide	CaM or CaM–peptide
–TnI peptide	$+Ca^{2+}$	31	16
+TnI peptide	$+Ca^{2+}$	30	15
–mastoparan	$+Ca^{2+}$	32	16
+mastoparan	$+Ca^{2+}$	29	15

the $[\theta]_{222}$ value increases to –23 988° in a 50% solution of the helix-inducer TFE. This value indicates that 78% of the peptide or approximately 11 residues assume an α-helical conformation under these conditions. Comparison of the theoretical and observed spectra for the TnC–mastoparan complex indicates that formation of this complex involves the induction of α-helix. In the presence and absence of Ca^{2+}, the observed negative ellipticity increases suggest that 14 or 17 residues, respectively, become α-helical in order for the complex to form (Table II). Interestingly, the change in the ellipticity value at 222 nm of mastoparan in 50% TFE from its value under benign conditions and the changes in ellipticity generated by complex formation are quantitatively similar. This suggests the possibility that a significant fraction of the helix induction in the complex occurs in mastoparan. It is unlikely, however, that it all occurs there, since this would leave the induction of α-helix corresponding to three or six residues in the presence or absence of Ca^{2+}, respectively (Table I), unaccounted for. Hence, it is probable that complex formation involves helix induction in both TnC and mastoparan.

Figure 4 displays the CD spectra for CaM, mastoparan, and their complex. As was the case with TnC, it is clear from comparison of the theoretical and observed spectra for the complex that complex formation involves the induction of additional α-helical structure. However, in this case, the number of residues involved is significantly smaller, corresponding to eight or nine residues for the presence or absence of Ca^{2+}, respectively (Table II). On the basis of the data which follow, it is likely that most or perhaps all of the α-helix induced occurs in residues of mastoparan in this case with very few or none being located in CaM.

Induction of α-Helix in TnC and CaM by Ca^{2+} in the Presence and Absence of TnI Peptide and Mastoparan. Figures 1 and 2 display the CD spectra for TnC and CaM in the presence and absence of Ca^{2+}. It is clear from these spectra that the role of Ca^{2+} in stabilizing or forming α-helical structure is much more pronounced in TnC than in CaM. From the data in Figures 1 and 2, we can calculate that the ratio of the percent helix values for TnC compared to CaM is 0.72 in the absence of Ca^{2+}, and 0.90 in its presence. Hence, even though the two proteins exhibit nearly the same percentage of helical residues when Ca^{2+} is present, in its absence TnC has approximately 15% fewer. As a consequence of these and related observations from other laboratories (Leavis et al., 1978; Nagy et al., 1978; Nagy & Gergely, 1979; Tsalkova & Privalov, 1985), it is reasonable to presume that the majority of the additional α-helix induced by mastoparan binding to TnC compared to mastoparan binding to CaM corresponds to residues which are already helical in CaM in the absence of mastoparan. Identification of which particular residues in TnC are involved in helix induction in TnC by complex formation is more problematic.

Table II shows that the number of residues involved in induction of α-helix by Ca^{2+} in both TnC and CaM is relatively unaffected by complex formation with either mastoparan or TnI peptide. In the case of CaM, helix corresponding to approximately 15 residues is induced by Ca^{2+} regardless of the presence or absence of either of the peptides. For TnC, the amount of helix induced by Ca^{2+} is equivalent to approximately 30 residues, a value which also is nearly inde-

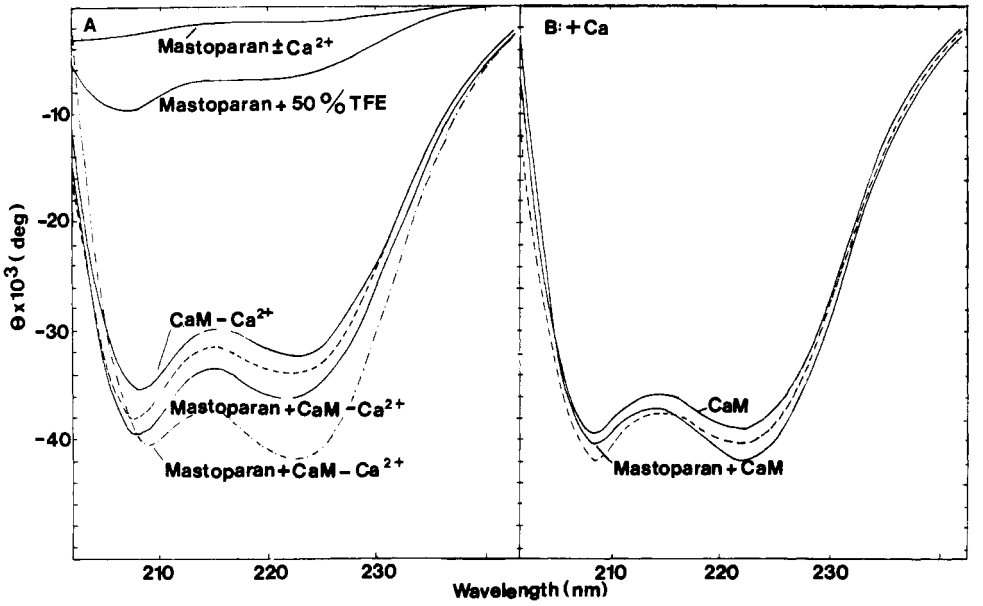

FIGURE 4: Far-ultraviolet circular dichroism spectra of mastoparan and CaM. (A) Spectra of mastoparan in 50% TFE and in the absence and presence of Ca^{2+}. In the absence of Ca^{2+}, the spectra of CaM and CaM–mastoparan (1:1) and the theoretical circular dichroism spectrum calculated for the mastoparan–CaM mixture (---) are shown. In the presence of Ca^{2+}, the spectrum of CaM–mastoparan (-·-) is shown. (B) Spectra of CaM and CaM–mastoparan (1:1) and the theoretical circular dichroism spectrum calculated for CaM–mastoparan (---) in the presence of Ca^{2+}. Buffers used contained 50 mM KCl, 20 mM NH_4HCO_3, and 1 mM EGTA at pH 7.3 (minus Ca^{2+}) or same plus 2.4 mM $CaCl_2$ (plus Ca^{2+}).

pendent of complex formation with the two peptides. This suggests that many of the residues involved in helix induction in TnC by mastoparan or TnI inhibitory peptide binding are different from those which are concerned with helix formation by Ca^{2+}, the one notable exception to this being that helix corresponding to three more residues is induced in the TnC–mastoparan complex in the absence of Ca^{2+} than in its presence, suggesting that Ca^{2+} binding and mastoparan binding induce α-helix in at least three of the same residues. Alternatively, it is possible that when the complex forms in the absence of Ca^{2+}, fewer residues are involved in helix induction in mastoparan while more are induced in TnC than is the case when Ca^{2+} is present. If this does occur, it would mean that more than three residues can be involved in helix induction by either Ca^{2+} or mastoparan.

To examine this matter further, we investigated the effect of the helix-inducing solvent TFE on TnC and CaM in the presence and absence of Ca^{2+}. It is clear from the data in Table I that TFE induces additional α-helical structure in both proteins. Significantly, in the case of CaM, the induction of α-helix by TFE has no effect on the quantity of α-helix induced by Ca^{2+} since an increase in $[\theta]_{222}$ of approximately −2800° occurs upon Ca^{2+} addition regardless of the order of addition. This strongly suggests that TFE and Ca^{2+} induce α-helix in different regions of CaM. The situation with TnC appears to be more complicated. Fifty percent TFE can induce approximately 94% of the amount of α-helix that Ca^{2+} does. Despite, this, the addition of Ca^{2+} to TnC already in the TFE solution causes a further large increase corresponding to a final helix for TnC or nearly 70% which is about 10% higher than the value for TnC plus Ca^{2+} alone. However, it is also worth noting that the increase in negative ellipticity at 222 nm caused by Ca^{2+} addition when 50% TFE is present is only 51% as large as the increase measured in the absence of TFE. These observations suggest that Ca^{2+} and TFE are capable of inducing helix in many of the same residues as well as various different ones. Comparison of the approximate numbers of residues induced into α-helical form under the different conditions indicates that between 16 and 20 residues may participate in helix induction by either TFE or Ca^{2+} while helix corresponding to 12 or 17 residues is induced specifically by TFE and Ca^{2+}, respectively. In reference to our earlier observations, we conclude from this that peptide binding can promote α-helix formation both in regions of TnC which are affected by Ca^{2+} and also in regions which are not affected.

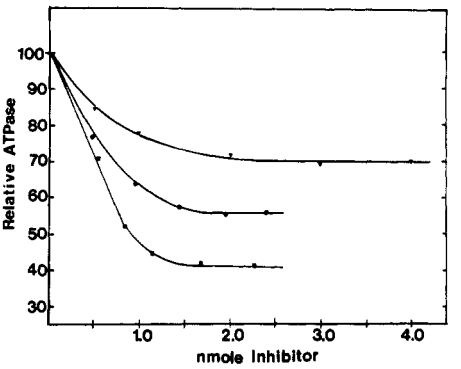

FIGURE 5: Inhibition of acto–S1–TM ATPase activity by TnI, TnI inhibitory peptide, and mastoparan. The acto–S1–TM ATPase activity was taken to be 100. The ATPase activity measurements were carried out with the continuous titration method. The S1, actin, and TM concentrations were 3.1, 1.64, and 0.30 μM, respectively, giving a molar ratio of 10.3:5.5:1. At 0.6 nmol of inhibitor in the assay, there is a 1:1 ratio of inhibitor to TM. (■) TnI; (●) TnI inhibitory peptide; (▼) mastoparan.

Inhibition of the Acto–S1–TM ATPase by Mastoparan, TnI Peptide, and TnI. Since mastoparan, like TnI peptide, binds to TnC, and since TnI peptide also binds to F-actin–TM and inhibits the acto–S1–TM ATPase (Talbot & Hodges, 1979, 1981a,b), it is reasonable to propose that mastoparan may also perform the latter function on an in vitro system. Figure 5 displays relative acto–S1–TM ATPase rates as a function of added protein or peptide. As expected from previous work in our laboratory, both the TnI inhibitory peptide and TnI exhibited substantial inhibitory activity with percentage decreases in the ATPase activity of 45% and 59%, respectively (Talbot & Hodges, 1979, 1981a). Significantly, addition of mastoparan to the assay results in a 30% inhibition of the ATPase

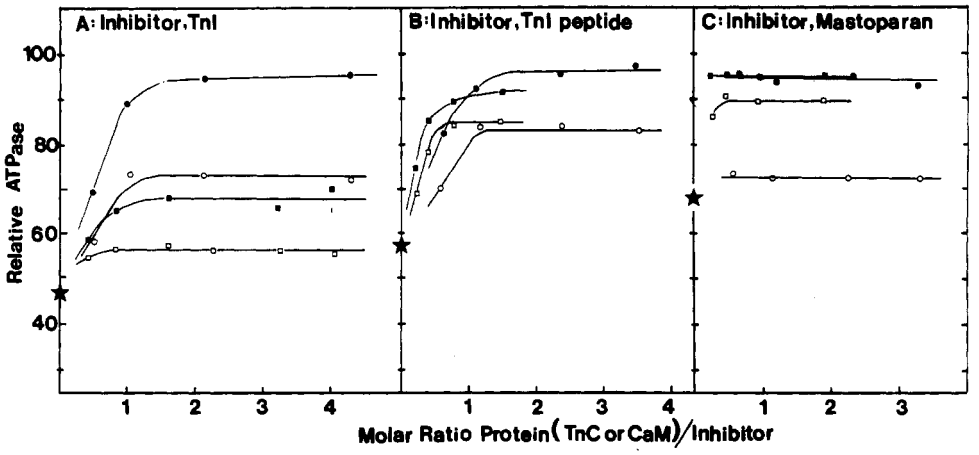

FIGURE 6: Effect of TnC and CaM in the absence and presence of Ca^{2+} on release of acto-S1-TM ATPase inhibition by TnI, TnI inhibitory peptide, or mastoparan. The acto-S1-TM ATPase activity was taken to be 100. The ATPase activity measurements were carried out by using the multiple table assay. The S1, actin, and TM concentrations were 3.1, 1.64, and 0.3 μM, respectively. (★) average acto-S1-TM ATPase activity induced by 1.5 nmol of TnI (panel A), TnI inhibitory peptide (panel B), and mastoparan (panel C); ATPase activity in the presence of the protein TnC in the absence (○) and presence of (●) of Ca^{2+} and the protein CaM in the absence (□) and presence (■) of Ca^{2+}.

value. Moreover, inhibition by mastoparan requires the presence of TM. In the absence of TM, the maximum inhibition achieved was 20–25% in all cases studied. Furthermore, without TM, maximum inhibition is delayed and takes effect at approximately 2–3 equiv for both TnI and TnI peptide. Mastoparan, however, did not reach its maximum inhibition over the range studied (0–3 equiv) when TM was absent. This is important because nonspecific proteins such as salmine, lysozyme, and cytochrome *c* have been shown to inhibit the acto-S1 ATPase in a manner which results in less inhibition when TM is present and which is presumably significantly different in mechanism than inhibition by the troponin-TM system (Syska et al., 1976; Talbot & Hodges, 1979). We interpret this observation as indicating that despite differences in sequence and conformation, mastoparan and TnI inhibitory peptide exhibit binding interfaces which have enough similar features that they are recognized by F-actin-TM as well as by TnC and CaM.

Ca^{2+}-Sensitive Release of Inhibition by TnC and CaM. The results shown in Figure 6 were obtained in experiments conducted to evaluate both the inhibitory ability of TnI, TnI inhibitory peptide, and mastoparan and the Ca^{2+}-sensitive release of inhibition by TnC and CaM. From panel A, it is apparent that TnC is much more effective at releasing inhibition by TnI than is CaM. In the absence of Ca^{2+}, CaM causes the ATPase activity to increase from a maximally inhibited value of 49% to a value of 57%. The presence of Ca^{2+} causes a further increase to only 68%. In contrast, TnC raises the relative ATPase value to 73% in the absence of Ca^{2+} and 95% in its presence. In light of the observation that TnI and TnC share at least two binding interfaces (Syska et al., 1976; Grabarek et al., 1981), we interpret the greater effectiveness of TnC at releasing inhibition to the likelihood that CaM does not interact as effectively as does TnC with TnI at the various binding interfaces. This interpretation is supported by the observation that Lys-18 of TnI is susceptible to covalent modification in the CaM-TnI complex but not in the TnC-TnI complex (Mohr et al., 1983).

Interestingly, the results for inhibition by TnI inhibitory peptide in panel B are rather different. In this case, the addition of CaM increases the ATPase activity from a maximally inhibited value of 58% to 85% in the absence of Ca^{2+} and 92% in its presence. For TnC, values of 83% and 96% are measured for the absence and presence of Ca^{2+}, respectively. However, despite the fact that TnC can provoke a greater release of inhibition than CaM when Ca^{2+} is present, it is clear that CaM is much more effective at low molar ratios than TnC at releasing inhibition regardless of Ca^{2+}. We believe that this observation is due to a higher degree of complex formation with the TnI peptide by CaM as a consequence of the 10-fold larger association constant for this complex compared to the complex formed with TnC (Malencik & Anderson, 1984).

Panel C of Figure 6 contains the data for inhibition by mastoparan. Without TnC or CaM present, the maximum relative ATPase value observed is 69%. The addition of TnC to the assay in the absence of Ca^{2+} raises this value only to 73%, whereas a value of 94% is measured when Ca^{2+} is present. Similarly, in the presence of Ca^{2+}, CaM induces nearly complete release of inhibition, 95% ATPase activity. However, in contrast to TnC, CaM also causes a relatively large increase in ATPase activity in the absence of Ca^{2+}, to 90% of the completely uninhibited value.

DISCUSSION

It has recently been hypothesized by Cox et al. (1985) that the minimal structural feature required of a high-affinity ligand for CaM is a basic amphiphilic α-helix approximately 15 Å or 2.8 helical turns in length. In this study, we have used circular dichroism to test this hypothesis by investigating the interactions of two ligands, mastoparan and TnI inhibitory peptide, with CaM and its close relative TnC. Our results support the contention that the structural features mentioned can lead to strong binding. However the α-helical feature is by no means a prerequisite.

The two ligands used were chosen for the following reasons. The interaction between the TnI inhibitory peptide and TnC both is physiologically relevant and has been well characterized by a variety of techniques which indicate that the peptide binds to the III_N helical region of TnC, and by analogy based on sequence homology and fragment binding studies to the same region of CaM [for reviews, see Leavis & Gergely (1984) and Cachia et al. (1985)]. Furthermore, TnI has been used as a model protein to study binding to CaM by Keller et al. (1982) and Olwin et al. (1982), and association constants for the interaction between the TnI inhibitory peptide and TnC and CaM in the presence of Ca^{2+} have been obtained (Malencik & Anderson, 1984). In contrast, the interaction between

mastoparan, the cytotoxic tetradecapeptide from the vespid wasp, and either CaM or TnC is not known to be physiologically relevant. However, mastoparan and several related bee or wasp venom peptides form stoichiometric complexes with CaM which exhibit surprisingly large association constants and produce inhibition of CaM-stimulated cAMP-dependent phosphodiesterase activity (Malencik & Anderson, 1983; Barnette et al., 1983). Mastoparan, for example, has association constants of 3.3×10^9 and 5.0×10^6 M^{-1} for CaM and TnC, respectively, when Ca^{2+} is present (Malencik & Anderson, 1983). Hence, mastoparan binds even more tightly to TnC and CaM than does TnI inhibitory peptide which has corresponding association constants of 1.1×10^6 and 1.0×10^5 M^{-1}, respectively (Malencik & Anderson, 1984). A consequence of this tight binding interaction has been that these venom peptides have been proposed as a model system for studying protein interactions with CaM. The bee venom peptide melittin, which has an association constant of 1×10^{10} M^{-1} for CaM in the presence of Ca^{2+}, has been used in this manner with interesting results (Malencik & Anderson, 1984). Maulet and Cox (1983) demonstrated that the α-helical content of melittin increased from 5% to 72% when it bound to CaM in the presence of Ca^{2+}. Interestingly, melittin along with mastoparan, mastoparan X, and *Polestes* mastoparan all have the sequence properties of basic amphiphilic helices, i.e., an α-helix in which the hydrophobic and hydrophilic residues are located on opposite sides (McDowell et al., 1985). In aqueous solution, melittin and mastoparan exhibit relatively little ordered secondary structure. However, melittin converts to a highly helical form when it binds to phospholipid surfaces (Dufource & Faucon, 1977; Dawson et al., 1978; Drake & Hider, 1979) or CaM (Maulet & Cox, 1983) or when it is dissolved in either organic/water cosolvents (McDowell et al., 1985) or high ionic strength solutions (Drake & Hider, 1979). Similarly, Higashijima et al. (1983) found that mastoparan contained 60% or more α-helix when dissolved in methanol or bound to phospholipid membrane, while McDowell et al. (1985) observed that mastoparan contained 15% α-helix in dilute aqueous solution and 55% α-helix in a 70% solution of the helix-inducer TFE. Significantly, they have also shown that mastoparan X and *Polistes* mastoparan undergo conversion from 6% α-helix in aqueous solution to 61% and 51% α-helix, respectively, when bound to calmodulin and to 56% and 72% α-helix, respectively, when dissolved in 70% TFE, which suggests that mastoparan would be similarly affected by binding to CaM.

It is clear from our results that mastoparan, like melittin, mastoparan X, and *Polistes* mastoparan, undergoes conversion from a conformation which is low in α-helix to a highly α-helical form upon binding to TnC and CaM. If it is assumed that no α-helix is induced in CaM upon mastoparan binding, the calculated change in percentage helix for mastoparan would be increase from approximately 25% to 80%, which is consistent with the value of 78% observed when mastoparan is dissolved in 50% TFE. This corresponds to a total of three α-helical turns in the bound mastoparan which is in agreement with the hypothesis that approximately 2.8 helical turns would be required (Cox et al., 1985). It is clear that in the case of mastoparan binding to TnC, α-helix is induced in both the ligand and the protein since it would be impossible for helix corresponding to 14 or 17 additional residues to be induced in a tetradecapeptide already containing approximately 4 helical residues. Significantly, this is quite consistent with current knowledge of the III_N helical region of TnC (see below).

Table III: Primary Sequences of TnI Inhibitory Peptide and Mastoparan

TnI inhibitory peptide [104–115][a]	Ac-Gly-*Lys*-Phe-*Lys*-*Arg*-Pro-Pro-Leu-*Arg*-*Arg*-Val-*Arg*-amide
mastoparan[b]	Ile-Asn-Leu-*Lys*-Ala-Leu-Ala-Ala-Leu-Ala-*Lys*-*Lys*-Ile-Leu-amide

[a] Sequence is from Cachia et al. (1983). [b] Sequence is from Malencik and Anderson (1983).

In the absence of divalent metal ions, the C-terminal half of TnC containing the Ca^{2+}–Mg^{2+} sites is much less stable and contains significantly less α-helical structure than does the N-terminal half (Leavis et al., 1978; Johnson & Potter, 1978; Tsalkova & Privalov, 1980). Spectroscopic studies on whole TnC and synthetic peptides and CNBr fragments containing all or part of binding site III indicate that a significant fraction of the α-helix induced in TnC upon Ca^{2+} binding corresponds to induction of the III_N helix (Nagy et al., 1978; Nagy & Gergely, 1979; Reid et al., 1981). Our observation that more α-helix is induced in the TnC–mastoparan complex than can be accounted for by mastoparan alone strongly suggests that mastoparan binding, like Ca^{2+}, induces or stabilizes this region of TnC into an α-helical form. This conclusion is consistent with previous studies in which the antipsychotic drug trifluoperazine was found to induce α-helix upon binding to the III_N region of a synthetic peptide corresponding to binding site III (Gariépy & Hodges, 1983; Reid et al., 1983). In addition, it is supported by the fact that the binding of Ca^{2+} to CaM or TnC is strongly coupled energetically to the binding of mastoparan as well as a wide variety of other peptides, proteins, and antipsychotic drugs (Weiss & Levin, 1978; Olwin et al., 1982; Malencik & Anderson, 1982; Maulet & Cox, 1983; Ingraham & Swenson, 1984). Interpreted according to the theory of ligand binding developed by Weber (1972), the physical basis for this phenomenon is that Ca^{2+} binding to TnC or CaM stabilizes a conformational state of the protein which is closely related to the state which binds the corresponding peptide etc. with highest affinity. We attribute the apparent lack of significant helix formation in CaM upon mastoparan binding to the inherently greater stability of the α-helical structure in this protein as evidenced by differential scanning calorimetric results (Tsalkova & Privalov, 1985) and the data in Table I showing that CaM contains approximately 15% more α-helix than does TnC when Ca^{2+} is absent.

In sharp contrast to the situation with mastoparan, the TnI inhibitory peptide does not take on an α-helical conformation when bound to CaM or TnC. Comparison of the sequences of these two peptides shown in Table III helps to elucidate why both peptides bind, as well as the reason why they bind in different conformations. Significantly, both peptides contain a number of basic residues. This characteristic has been previously observed in a number of other CaM binding peptides (Malencik & Anderson, 1982, 1983a,b; Cox et al., 1985), and the presence of a positive charge is also important in the binding of phenothiazine and related drugs (Prozialeck & Weiss, 1982). Malencik and Anderson (1982), for example, observed a pattern for peptides which bound CaM well which consisted of a strongly basic tripeptide sequence three residues away from a pair of bulky hydrophobic residues. Sequence alignment based on conservative replacement of the basic residues shows that all three basic residues in mastoparan have corresponding basic residues on the TnI inhibitory peptide when the sequences are aligned. This is notable since a previous study using synthetic analogues of the latter peptide has shown that residues Lys-105 and Arg-113 contribute sub-

stantially to the ability of the peptide to inhibit the acto–S1–TM ATPase (Talbot & Hodges, 1981). This suggests that the corresponding basic residues in mastoparan may be involved in the ability of mastoparan to inhibit the acto–S1–TM ATPase as well as bind to TnC and CaM. Further comparison of the aligned sequences reveals that the pattern noted by Malencik and Anderson (1982) is observed with mastoparan but is only partially observed in the case of TnI inhibitory peptide since with the latter a basic dipeptide, Lys-107 and Arg-108, is found three residues away from the basic tripeptide instead of a hydrophobic dipeptide. Considering the demonstrated importance of other basic residues in the TnI inhibitory peptide and the fact that this basic dipeptide is conserved in four different species of TnI (Wilkinson & Grand, 1978), it is probable that they play an important role in the ability of TnI to bind to TnC properly.

In light of our experimental results, it was of particular interest to compare the sequences using the secondary structure prediction method of Chou and Fasman (1974). According to this method, TnI inhibitory peptide has no tendency to form either a α-helix or a β-strand. Further, any α-helix which might form would definitely be disrupted by the two Pro residues in the middle of the sequence. As a consequence of these Pro residues, residues 109–112 could form a reverse turn as has been pointed out by Wilkinson and Grand (1978). In order to evaluate this latter possibility, Talbot and Hodges (1981b) synthesized an analogue of the peptide in which Pro-110 was replaced by Thr as it is in rabbit cardiac TnI. Despite the fact that this lowered the reverse-turn-forming potential by 65%, no significant loss in the inhibitory ability of this peptide was observed. This suggests that the likelihood that TnI inhibitory peptide contains a reverse turn upon binding is minimal. In contrast, mastoparan was predicted to have a high α-helical propensity with only one helix-breaking residue, Asn-13. This prediction is quite consistent with our measurement of 12 α-helical residues for mastoparan in 50% TFE. Hence, this secondary structure prediction method predicts and our experimental data demonstrate that when TnI inhibitory peptide and mastoparan bind to TnC and CaM, they exhibit different secondary structures.

Since the interaction between TnI inhibitory peptide and TnC is physiologically relevant and that between mastoparan and CaM is not, our conclusions cast a significant degree of doubt upon the suitability of using basic amphiphilic helices as a general model for describing CaM–enzyme interactions. Additional observations which are pertinent to this matter concern the affinities of CaM and TnC for various substrates and the question of how these substrates bind. Mastoparan binds to CaM with a K_d value of 0.3 nM. This value is quite close in magnitude to K_d and K_m values measured for the interactions between CaM and various target enzymes such as myosin light chain kinase (K_m = 1.3 nM) (Malencik et al., 1982), phosphorylase kinase ($K_d = K_m$ = 10 nM) (Picton et al., 1980; Kuzicki et al., 1981), and cyclic nucleotide phosphodiesterase (K_m = 1 nM) (Kuzicki et al., 1981). Similarly, the K_d for the TnC–TnI interaction is 0.6 nM (Ingraham & Swenson, 1984). It is partially on the basis of the similar binding constants exhibited by these physiologically relevant interactions with the CaM–mastoparan and related interactions with bee and wasp venom peptides which have led to the conclusion that basic amphiphilic peptides provide a good model for these interactions (Maulet & Cox, 1983; Cox, 1984). This conclusion, however, neglects the fact that target enzymes such as cyclic nucleotide phosphodiesterase, cAMP-dependent protein kinase, and phosphorylase bind to CaM through multiple binding interactions involving two or more regions of the various molecules (Picton et al., 1980; Kuznicki et al., 1981; Ni & Klee, 1985). Similarly, TnI binding to TnC involves at least two different regions of each molecule (Syska et al., 1976; Grabarek et al., 1981). Consequently, in the case of the natural target proteins, the high affinity for these regulatory proteins is achieved through at least two different interactions. Hence, the individual microscopic K_d values which contribute to the overall interaction are larger than the observed K_d. For example, although the TnC–TnI interaction has a K_d of 0.6 nM (Ingraham & Swenson, 1984), the interaction between TnC and TnI inhibitory peptide is 10 μM (Malencik & Anderson, 1984). Since there is no evidence that an individual mastoparan molecule interacts with more than one region of CaM, we conclude that its small K_d value for CaM does not necessarily make all of the molecular aspects involved in its binding interactions with CaM representative of physiologically relevant ones.

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Registry No. TFE, 75-89-8; ATPase, 9000-83-3; Ca, 7440-70-2; TnI inhibitory peptide [104–115], 98353-69-6; mastoparan, 72093-21-1.

REFERENCES

Andersson, A., Forsén, S., Thulin, E., & Vogel, H. J. (1983) *Biochemistry 22*, 2309.

Aulabaugh, A., Niemczura, W. P., & Gibbons, W. A. (1984) *Biochem. Biophys. Res. Commun. 115*, 225.

Babu, Y. S., Sack, J. S., Greenhough, T. J., Bugg, C. E., Means, A. R., & Cook, W. J. (1985) *Nature (London) 315*, 37.

Brzeska, H., Szynkiewicz, J., & Drabikowski, W. (1983) *Biochem. Biophys. Res. Commun. 115*, 87.

Cachia, P. J., Sykes, B. D., & Hodges, R. S. (1983) *Biochemistry 22*, 4145.

Cachia, P. J., Gariépy, J., & Hodges, R. S. (1985) in *Calmodulin Antagonists and Cellular Physiology* (Hidaka, H., & Hartshorne, D. J., Eds.) pp 63–88, Academic Press, New York.

Chang, C. T., Wu, C. H. C., & Yang, J. T. (1978) *Anal. Biochem. 91*, 13.

Chong, P. C. S., & Hodges, R. S. (1981) *J. Biol. Chem. 256*, 5064.

Chong, P. C. S., Asselbergs, P. J., & Hodges, R. S. (1983) *FEBS Lett. 153*, 372.

Chou, P. Y., & Fasman, G. D. (1974) *Biochemistry 13*, 211.

Collins, J. H., Potter, J. D., Horn, M. J., Wilshire, G., & Jackman, N. (1973) *FEBS Lett. 36*, 268.

Cox, J. A. (1984) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 43*, 3000.

Cox, J. A., Comte, M., Fitton, J. E., & DeGrado, W. F. (1985) *J. Biol. Chem. 260*, 2527.

Dawson, C. R., Drake, A. F., Helliwell, J., & Hider, R. C. (1978) *Biochim. Biophys. Acta 510*, 75.

Dedman, J. R., Jackson, R. L., Schreiber, W. E., & Means, A. R. (1978) *J. Biol. Chem. 253*, 343.

Drake, A. F., & Hider, R. C. (1979) *Biochim. Biophys. Acta 555*, 371.

Gariépy, J., & Hodges, R. S. (1983) *Biochemistry 22*, 1586.

Gariépy, J., Sykes, B. D., Reid, R. E., & Hodges, R. S. (1982) *Biochemistry 21*, 1506.

Giedroc, D. P., Puett, D., Ling, N., & Staros, J. V. (1983) *J. Biol. Chem. 258*, 16.
Gopalakrishan, R., & Anderson, W. B. (1982) *Biochem. Biophys. Res. Commun. 104*, 830.
Grabarek, Z., Drabikowski, W., Leavis, P., Rosenfield, S. S., & Gergely, J. (1981) *J. Biol. Chem. 256*, 13121.
Guerini, D., Krebs, J., & Carafoli, E. (1984) *J. Biol. Chem. 259*, 15172.
Herzberg, O., & James, M. N. G. (1985) *Nature (London) 313*, 653.
Higashijima, T., Wakamatsu, K., Takemitsu, M., Fujino, M., Nakajima, T., & Miyazawa, T. (1983) *FEBS Lett. 152*, 227.
Horowitz, J., Bullard, B., & Mercola, D. (1979) *J. Biol. Chem. 254*, 350.
Ingraham, R. H., & Swenson, C. A. (1984) *J. Biol. Chem. 259*, 9544.
Johnson, J. D., & Potter, J. D. (1978) *J. Biol. Chem. 253*, 3775.
Keller, C. H., Olwin, B. B., LaPorte, D. C., & Storm, D. R. (1982) *Biochemistry 21*, 156.
Klevit, R. E., & Vanaman, T. C. (1984) *J. Biol. Chem. 259*, 15414.
Klevit, R. E., Dalgarno, D. C., Levine, B. A., & Williams, R. J. P. (1984) *Eur. J. Biochem. 139*, 109.
Krebs, J., Buerkler, J., Guerini, D., Brunner, J., & Carafoli, E. (1984) *Biochemistry 23*, 400.
Kretsinger, R. H., & Nockolds, C. E. (1973) *J. Biol. Chem. 248*, 3313.
Kuznicki, J., Grabarek, Z., Brzeska, H., Drabikowski, W., & Cohen, P. (1984) *FEBS Lett. 130*, 141.
LaPorte, D. C., Wierman, B. M., & Storm, D. R. (1980) *Biochemistry 19*, 3814.
Leavis, P. C., Rosenfeld, S. S., Gergely, J., Grabarek, Z., & Drabikowski, W. (1978) *J. Biol. Chem. 253*, 5452.
Malencik, D. A., & Anderson, S. R. (1982) *Biochemistry 21*, 3480.
Malencik, D. A., & Anderson, S. R. (1983a) *Biochemistry 22*, 1995.
Malencik, D. A., & Anderson, S. R. (1983b) *Biochem. Biophys. Res. Commun. 114*, 50.
Malencik, D. A., & Anderson, S. R. (1984) *Biochemistry 23*, 2420.
Maulet, Y., & Cox, J. A. (1983) *Biochemistry 22*, 5680.
McDowell, L., Sanyal, G., & Prendergast, F. G. (1985) *Biochemistry 24*, 2979.
Moir, A. J. G., Ordidge, M., Grand, R. J. A., Trayer, I. P., & Perry, S. V. (1983) *Biochem. J. 209*, 417.
Nagy, B., & Gergely, J. (1979) *J. Biol. Chem. 254*, 12732.
Nagy, B., Potter, J. D., & Gergely, J. (1978) *J. Biol. Chem. 253*, 5971.
Newton, D. L., Oldewurtel, M. D., Krinks, M. H., Shiloach, J., & Klee, C. B. (1984) *J. Biol. Chem. 259*, 4419.
Ni, W. C., & Klee, C. B. (1985) *J. Biol. Chem. 260*, 6974.
Olwin, B. B., Keller, C. H., & Storm, D. R. (1982) *Biochemistry 21*, 5669.
Pato, M. B., Mak, A. S., & Smillie, L. B. (1981) *J. Biol. Chem. 256*, 593.
Picton, C., Klee, C. B., & Cohen, P. (1980) *Eur. J. Biochem. 111*, 553.
Potter, J. D., & Gergely, J. (1975) *J. Biol. Chem. 250*, 4628.
Prozialeck, W. C., & Weiss, B. (1982) *J. Pharmacol. Exp. Ther. 222*, 509.
Puett, D., Giedroc, D. P., Tollefson, S., & Ling, N. (1983) *Peptides (N.Y.) 4*, 191–194.
Reid, R. E., Gariépy, J., Saund, A. K., & Hodges, R. S. (1981) *J. Biol. Chem. 256*, 2742.
Reid, R. E., Gariépy, J., & Hodges, R. S. (1983) *FEBS Lett. 154*, 60.
Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866.
Syska, H., Wilkinson, J. M., Grand, R. J. A., & Perry, S. V. (1976) *Biochem. J. 153*, 375.
Talbot, J. A., & Hodges, R. S. (1979) *J. Biol. Chem. 254*, 3720.
Talbot, J. A., & Hodges, R. S. (1981a) *J. Biol. Chem. 256*, 2798.
Talbot, J. A., & Hodges, R. S. (1981b) *J. Biol. Chem. 256*, 12374.
Tsalkova, T. N., & Privalov, P. L. (1985) *J. Mol. Biol. 181*, 533.
Vanaman, T. C., Shariel, F., & Watterson, D. M. (1977) in *Calcium-Binding Proteins and Calcium Function* (Wasserman, R. H., et al., Eds.) pp 107–116, Elsevier, Amsterdam.
Watterson, D. M., & Vincenzi, F. F., Eds. (1980) *Ann. N.Y. Acad. Sci. 356*, 1.
Watterson, D. M., Harrelson, W. G., Keller, P. M., Sharief, F., & Vanaman, T. C. (1976) *J. Biol. Chem. 251*, 4501.
Weber, G. (1972) *Biochemistry 11*, 864.
Weeds, A. G., & Taylor, R. S. (1975) *Nature (London) 257*, 54.
Weiss, B., & Levin, R. M. (1978) *Adv. Cyclic Nucleotide Res. 9*, 285.
Wilkinson, J. M., & Grand, R. J. A. (1978) *Nature (London) 271*, 31.