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Model for the Interaction of Amphiphilic Helices With Troponin C and Calmodulin

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ABSTRACT **Crystals of troponin C are stabilized by an intermolecular interaction that involves the packing of helix A from the N-terminal domain of one molecule onto the exposed hydrophobic cleft of the C-terminal domain of a symmetry related molecule. Analysis of this molecular recognition interaction in troponin C suggests a possible mode for the binding of amphiphilic helical molecules to troponin C and to calmodulin. From the template provided by this troponin C packing, it has been possible to build a model of the contact region of mastoporan as it might be bound to the two Ca^{2+} binding proteins. A possible binding mode of melittin to calmodulin is also proposed. Although some of the characteristics of binding are similar for the two amphiphilic peptides, the increased length of melittin requires a significant bend in the calmodulin central helix similar to that suggested recently for the myosin light chain kinase calmodulin binding peptide (Persechini and Kretsinger: *Journal of Cardiovascular Pharmacology* 12:501–512, 1988). Not only are the hydrophobic interactions important in this model, but there are several favorable electrostatic interactions that are predicted as a result of the molecular modeling. The regions of troponin-C and calmodulin to which amphiphilic helices bind are similar to the regions to which the neuroleptic drugs such as trifluoperazine have been predicted to bind (Strynadka and James: *Proteins* 3:1–17, 1988).**

Key words: computer modeling, Ca^{2+}-binding proteins, hydrophobic binding interactions

INTRODUCTION

Troponin C (TnC) and calmodulin (CaM) belong to the helix-loop-helix (HLH) Ca^{2+}-binding protein family.[1] Each protein has four homologous HLH structural motifs that bind Ca^{2+} ions with K_d's ranging from 10^{-5} to 10^{-7} M.[2–5] The binding of calcium to the loops of the HLH units induces the conformational changes in TnC and CaM that are required for the optimal interactions with their target molecules. The effect of the conformational change in TnC is transmitted to the thin filament proteins troponin I, tropomyosin, and actin, thereby triggering muscle contraction (for recent reviews of the biochemical data, see refs. 2,3). CaM binds to and modulates the activities of a variety of diverse intracellular enzymes, including myosin light chain kinase (MLCK), calcineurin, erythrocyte Ca^{2+}-ATPase, brain adenylate cyclase, and cyclic 3',5' monophosphate phosphodiesterase.[4,5]

The high-resolution crystal structures of turkey TnC,[6] chicken TnC,[7] and bovine brain CaM,[8] have been determined. These crystal structures revealed dumbbell-shaped molecules having two globular domains (the N- and C-terminal domains) joined by a long helical linker (Fig. 1). Each globular domain is cup-shaped. From this analogy, the bottom of the cup contains the pair of Ca^{2+} binding loops, the outside and upper rim of the cup is composed mainly of negatively charged residues, and the interior of the cup is lined with hydrophobic residues (Fig. 2a,b). In the C-terminal domain of TnC and in both the N- and C-terminal domains of CaM, the Ca^{2+}-binding sites are occupied by metal ions; the resulting conformations of these domains are similar.[9] In them, the hydrophobic residues that line the interior of the cup are relatively exposed (Fig. 3a–c). However, due to the low pH of the crystallization conditions,[7,10] the N-terminal domain of TnC has no ions in the expected coordination sites and consequently adopts a very different tertiary arrangement.[11] Thus, in the absence of bound Ca^{2+}, the N-terminal domain is much more "closed" with the interior hydrophobic residues buried away from the protein/solvent interface (Fig. 3d).

Because of the similar conformations of the Ca^{2+}-filled domains of TnC and CaM and of the extensive homology between the N- and C-terminal domains of TnC, it seemed probable that the N-terminal domain of TnC should adopt a fold similar to that of the Ca^{2+}-filled state. A model for the interconver-

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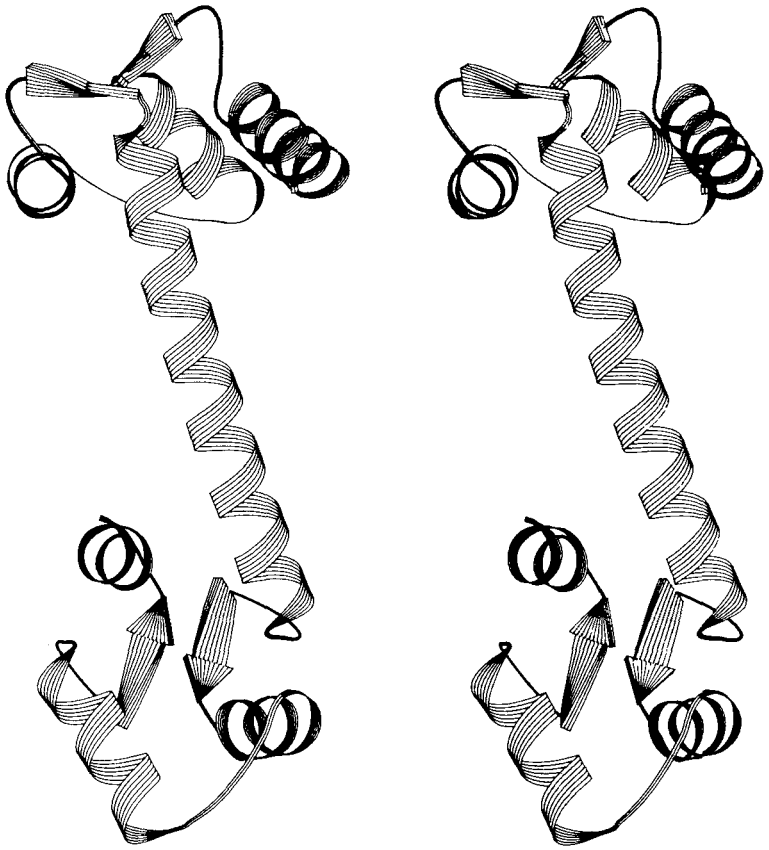

Fig. 1. A stereographic representation ribbon diagram of bovine brain CaM.[8] This view shows the prominent secondary structural features that form the N and C-terminal Ca^{2+} binding domains and the long helical linker joining them.

sion of the Ca^{2+}-free (closed) and Ca^{2+}-bound (open) forms of the N-terminal domain of TnC has been proposed.[11] The model is supported by a number of solution studies that implicate the movement of several hydrophobic residues to a more solvent exposed state upon calcium binding.[12–16]

The calcium-bound conformations of TnC and CaM are required for high affinity binding to target molecules.[2–5] Several studies indicate that the hydrophobic surfaces of TnC and CaM that become exposed upon calcium-binding form the sites of target molecule interactions.[13–26] Although the details of these interactions have not been defined at the molecular level, it is clear that both hydrophobic and electrostatic interactions are involved. Additionally, there is increasing evidence that amphiphilic helices may play a major role in target molecule binding. The proposed CaM binding sites on myosin light chain kinase,[27,28] phosphofructokinase,[29] phosphorylase b kinase[28,30] and calmodulin-dependent protein kinase from brain,[31] all encompass short sections of each enzyme that have high amphiphilic helix forming propensities. As well, many known potent inhibitors of CaM and TnC are short amphiphilic helical peptides (for a recent review see 32). Ostensibly, their inhibitory action is through competitive binding with target molecules for the exposed hydrophobic regions on TnC and CaM.

Analysis of the crystal packing of turkey skeletal TnC provides a possible model for the binding of amphiphilic helices to the hydrophobic clefts of TnC and CaM. The molecular packing of the native turkey skeletal TnC in the trigonal crystals shows that the A helix of one TnC molecule fits into the open Ca^{2+}-bound C-terminal hydrophobic cleft of a symmetry related molecule (Fig. 4). We have used this helix as a template for our modelling. The following describes our analysis of the modelled TnC and CaM complexes with mastoporan and melittin.

MATERIALS AND METHODS

The A helix (for helix nomenclature see ref. 9) of the molecule at $-x+y$, $1-x$, $z+1/3$ in the crystal structure of native turkey skeletal troponin C was used as a template for the modeling of amphiphilic peptides into the Ca^{2+}-bound hydrophobic cleft on the adjacent symmetry related neighbor at x,y,z. These molecules are denoted in Figure 4 as mole-

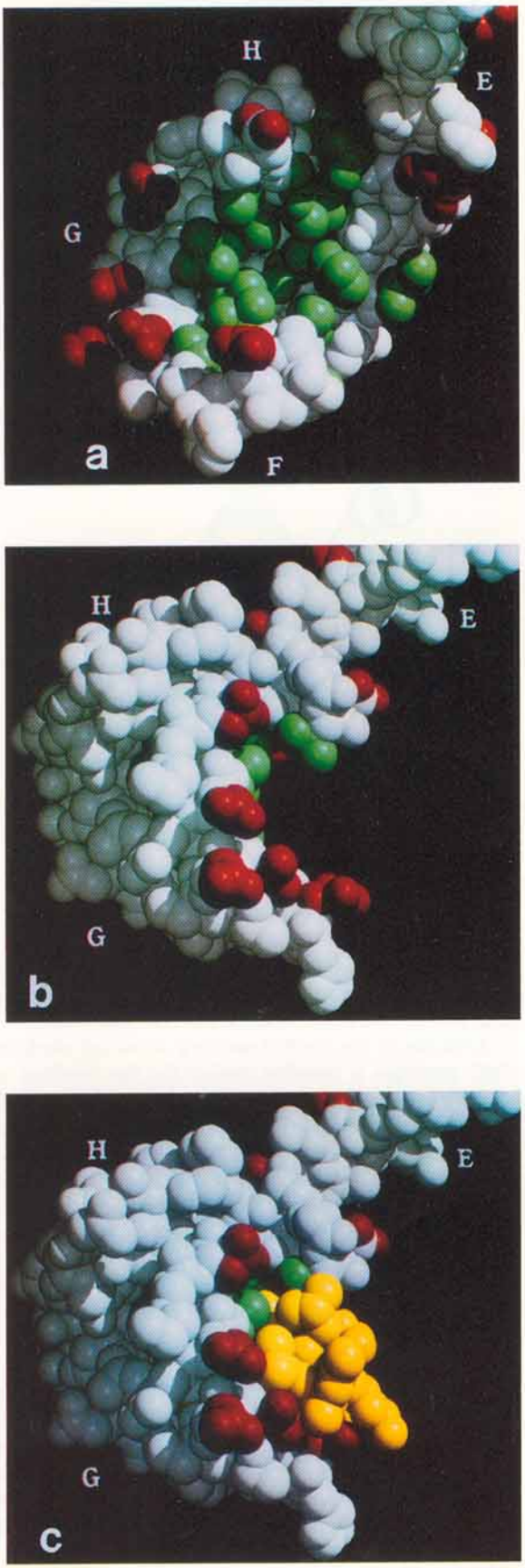

Fig. 2. **a:** A space-filling diagram of the C-terminus of turkey skeletal TnC looking directly into the C-terminal hydrophobic cup. Apolar residues are shown in green and acidic residues in red. Helices are labelled E, F, G, and H according to the nomenclature in reference 6. **b:** A view 90° to that in panel a, emphasizing the prominent hydrophobic cleft present in all the Ca^{2+} bound domains of TnC and CaM. **c:** A space-filling representation of the proposed TnC-mastoporan complex. The C-terminal domain of TnC is shown in an identical orientation to that in b. The mastoporan helix that neatly fills the pronounced hydrophobic cleft is shown in yellow.

cules 2 and 1, respectively. The program MUTATE (R. Read, unpublished) was used to substitute the appropriate residues of mastoporan[33] and melittin[34] into the TnC A-helix coordinate set. The sequence alignment used for these substitutions is given in Table I. Molecular surfaces to show van der Waal contacts were calculated using the algorithm of Lee and Richards.[35] Side chain clashes that violated van der Waals contact distances were minimized using the MMS suite of programs (S. Dempsey) on a Silicon Graphics Iris 3030 workstation.

The last three residues of the H helix in TnC are distorted from a helical conformation in the native structure due to crystal packing constraints (see Figs. 4, 5a). In order to deduce possible *interactions* that the H-helix of TnC might have with the modelled amphiphilic peptides, Glu159-Val161 were given a regular α-helical conformation, so that the H helix is continuous along its entire length.

For the modeling of the mastoporan and melittin interactions with bovine brain CaM, least-squares overlap methods were used to transform the A-helix coordinate set described above into both calcium saturated domains of the bovine brain calmodulin crystal structure.[8] Modeling then proceeded as detailed for TnC.

RESULTS AND DISCUSSION

Analysis of the Crystal Packing of Troponin C

Turkey skeletal TnC crystallizes in the trigonal space group $P3_221$ with unit cell dimensions a,b = 60.54 Å, c = 66.91 Å, γ = 120°.[6] There are six TnC molecules within the unit cell. The packing interaction of interest here involves the contacts between the molecule at x,y,z and the symmetry related one at $-x+y$, $1-x$, $z+1/3$. Figures 4 and 5a and the data in Table II clearly show that the A helix in the N-terminal domain of the molecule at $-x+y$, $1-x$, $z+1/3$ (molecule 2 in Fig. 4) fits neatly into the C-terminal hydrophobic pocket of the molecule at x,y,z. The C-terminal hydrophobic patch, formed by residues on helices E, F, the F-G linker, and helices G and H is in the Ca^{2+}-filled or "open" conformation in the native structure, i.e. its hydrophobic residues are relatively exposed to solvent (Fig. 3a). In general, large areas of solvent-exposed hydrophobic side chains are considered energetically unfavorable. Perhaps the packing of the relatively uncharged

face of the A-helix into the exposed hydrophobic pocket serves to minimize this unfavorable energy in the crystallization process. It is also tempting to speculate that this interaction may be responsible for the pH-dependent dimerization of TnC molecules in solution. Aggregation of TnC occurs only at acidic pH's that also prohibits metal binding to the low affinity N-terminal Ca^{2+}-binding sites.[36] This would suggest that the 2 Ca^{2+} form is required for dimerization to occur. Furthermore, the three-dimensional structures of turkey and chicken TnC show that under the low pH of the crystallization conditions (~4.8), the protonation of the carboxylate groups of acidic side chains allows for several strong carboxyl/carboxylate interactions which not only stabilize the Ca^{2+} free form of the N-terminus within each molecule, but also stabilize the interaction of the two-symmetry-related molecules shown in Figure 4.

Analysis of the X-Ray Crystallographically Defined Interaction of the A-Helix With the C-Terminal Domain of Troponin C

Hydrophobic interactions that stabilize the A-helix within the environment of the neighboring C-terminal hydrophobic cleft can be summarized as follows: The A-helix presents a relatively hydrophobic face to the adjacent apolar pocket (Ala20, Ala24, Ala25, Met28; see Fig. 5a; Table I). Because of the small surface area of alanine side chains, most of these nonpolar interactions are probably weak. The Cβ of Ala20 faces in towards the hydrophobic pocket where it is close to Leu122, Val129, and the Cβ of Glu127. Ala24 Cβ makes contact with the side chains of Phe105, Ile121, and Leu122. The Ala24 contact distances are quite long and observation of the calculated molecular surfaces shows the presence of a cavity in this region. By far the most extensive intermolecular hydrophobic interactions arise from Met28 of the A-helix. Met28 fits snugly into a pocket formed by Phe105 and Ile104. Met28 is also stabilized by interactions with Phe29 from the A-helix itself. All other hydrophobic residues of the A-helix (Table I) lie on the opposite face where they form intramolecular hydrophobic interactions within the N-terminal hydrophobic domain of their own molecule.

The packing of the A-helix into the neighboring C-terminal domain is also enhanced by several electrostatic interactions which occur along the rim of the hydrophobic cup. Glu16 on the A-helix forms a carboxyl-carboxylate interaction with the side chain of Glu127 on the F-G linker (Glu16 OE1-Glu127 OE2, 2.3 Å) of the symmetry-related molecule. In addition, a network of water molecules interacts with both Glu16 and Glu127 (Glu16 OE1-Wat197, 3.3 Å; Glu127 OE1-Wat205, 2.8 Å). As well, Glu17 forms a carboxyl-carboxylate interaction with Glu133* on the G-helix (Glu17 OE1-Glu133 OE1, 3.3 Å).

On the whole, the A-helix provides a weak hydrophobic face to the adjacent apolar cleft and the binding is also stabilized by electrostatic interactions on the rim of the cleft. A glaring exception to note is the side chain of Glu21 on the A-helix which points directly at the apolar pocket. To offset this potentially unfavorable interaction, an elaborate system of water molecules surrounding the negatively charged side chain probably serves to keep it relatively happy in its hydrophobic surroundings. Glu21 OE2 interacts with Wat261 (3.2 Å), in turn Wat261 forms a hydrogen bond with Wat227 (2.2 Å), and Wat227 contacts Wat199 (3.3 Å) (see Fig. 5a).

Analysis of the Modeled Interaction of Mastoporan With the C-Terminal Domain of Troponin C

TnC and CaM bind a variety of naturally occurring extracellular peptides, including the opiates β-endorphin[37] and dynorphin,[38] various members of the glucagon family[39,40] and a group of cytotoxic peptides, among them bee venom melittin[34,41,42] and the mastoporans[38,43] (Table I). Binding of these peptides to calcium saturated TnC and CaM is typically with high affinity ($K_d = 10^{-8} - 10^{-9}$ M) and has been shown to inhibit effectively the modulating activity of these two HLH proteins for their intracellular targets.[40,41,43–47,49]

There are two characteristics common to all the above TnC and CaM inhibitory peptides. Firstly, all contain a sequence with high α-helix forming propensity[48] (Table I). As well, the hydrophobic and hydrophilic residues are arranged in the sequence such that hydrophobic residues would extend off one face of the helix, and charged, hydrophilic residues from the opposite face; i.e., they are amphiphilic in nature. Solution circular dichroism and NMR studies involving β-endorphin,[40] mastoporan,[40,43,50,51] and melittin[42,44,52] indicated an induction of helical conformations in these peptides as they formed complexes with the complementary surface of CaM. Direct evidence for the amphiphilic helical nature of melittin comes from the X-ray crystallographic structure.[53] Finally, DeGrado and coworkers have designed from first principles short amphiphilic helices which bind CaM with submicromolar dissociation constants.[32,48] One particular peptide was synthesized with an environmentally sensitive fluorescent amino acid (Trp) sequentially placed at each position along the chain. The pattern of periodic fluorescence decay of this set of analogues in

*The final electron density map of turkey TnC is more consistent with a glutamate at position 133 rather than aspartate as in chicken. This has been confirmed by amino acid sequence studies (M.R. Carpenter, C.K. Golosinki & L.B. Smillie, unpublished data).

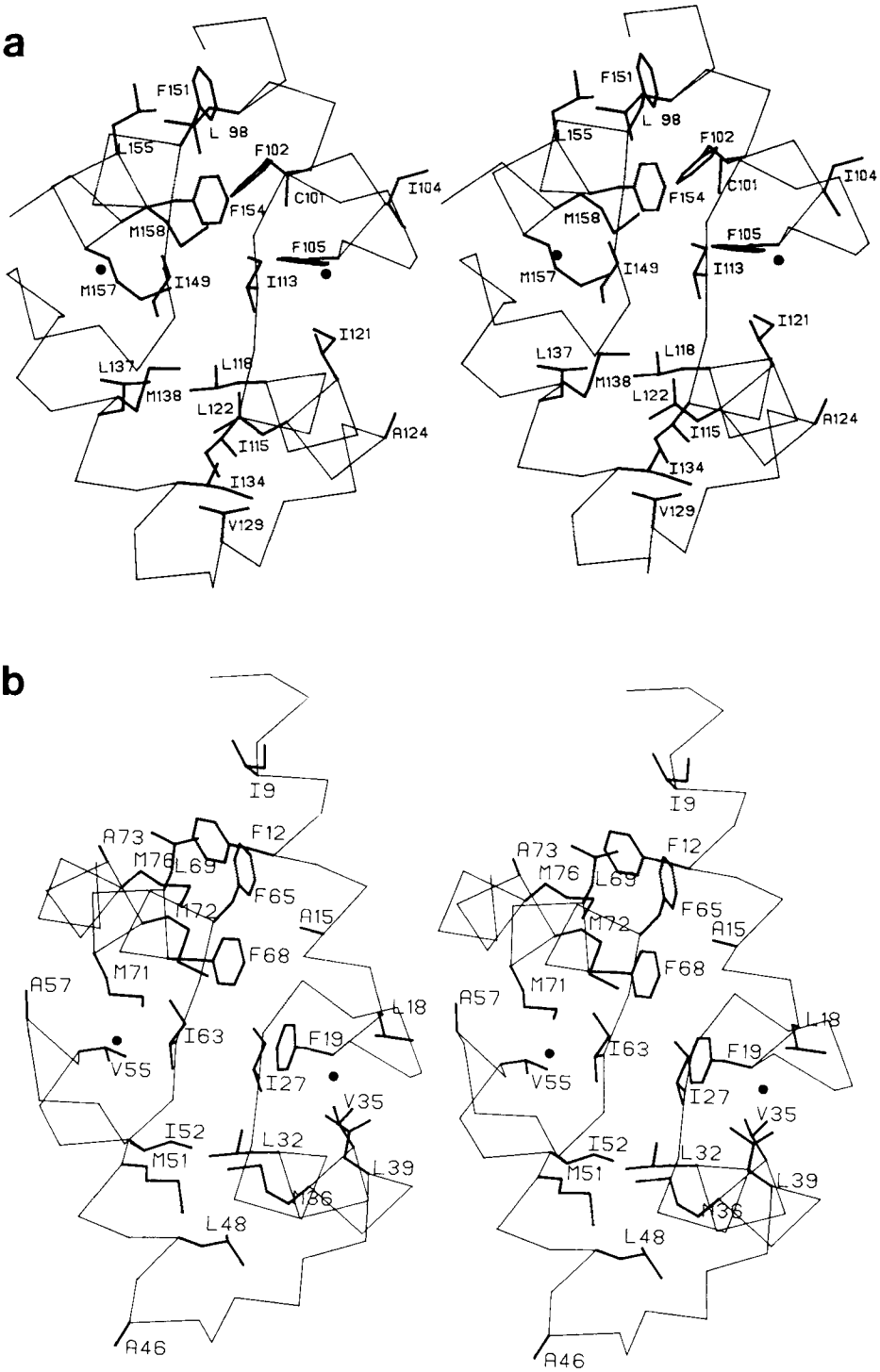

Fig. 3. Stereographic representations of the hydrophobic pockets of TnC and CaM. For clarity, only the hydrophobic side chains superimposed on the Cα-tracing are shown. Calciums are small black circles. All views are looking directly into the hydrophobic cup as in Figure 2a. **a:** The Ca^{2+}-bound C-terminal domain of TnC. **b:** The Ca^{2+}-bound N-terminal domain of CaM. **c:** The Ca^{2+}-bound C-terminal domain of CaM. **d:** The Ca^{2+}-free N-terminal domain of TnC.

complex with CaM could be explained by an α-helical structure for the bound peptide.[54]

If, as all the above evidence suggests, the peptides in Table I are amphiphilic helices, then it is clear that they would form a significant hydrophobic area, and thus, in turn would require a large complemen-

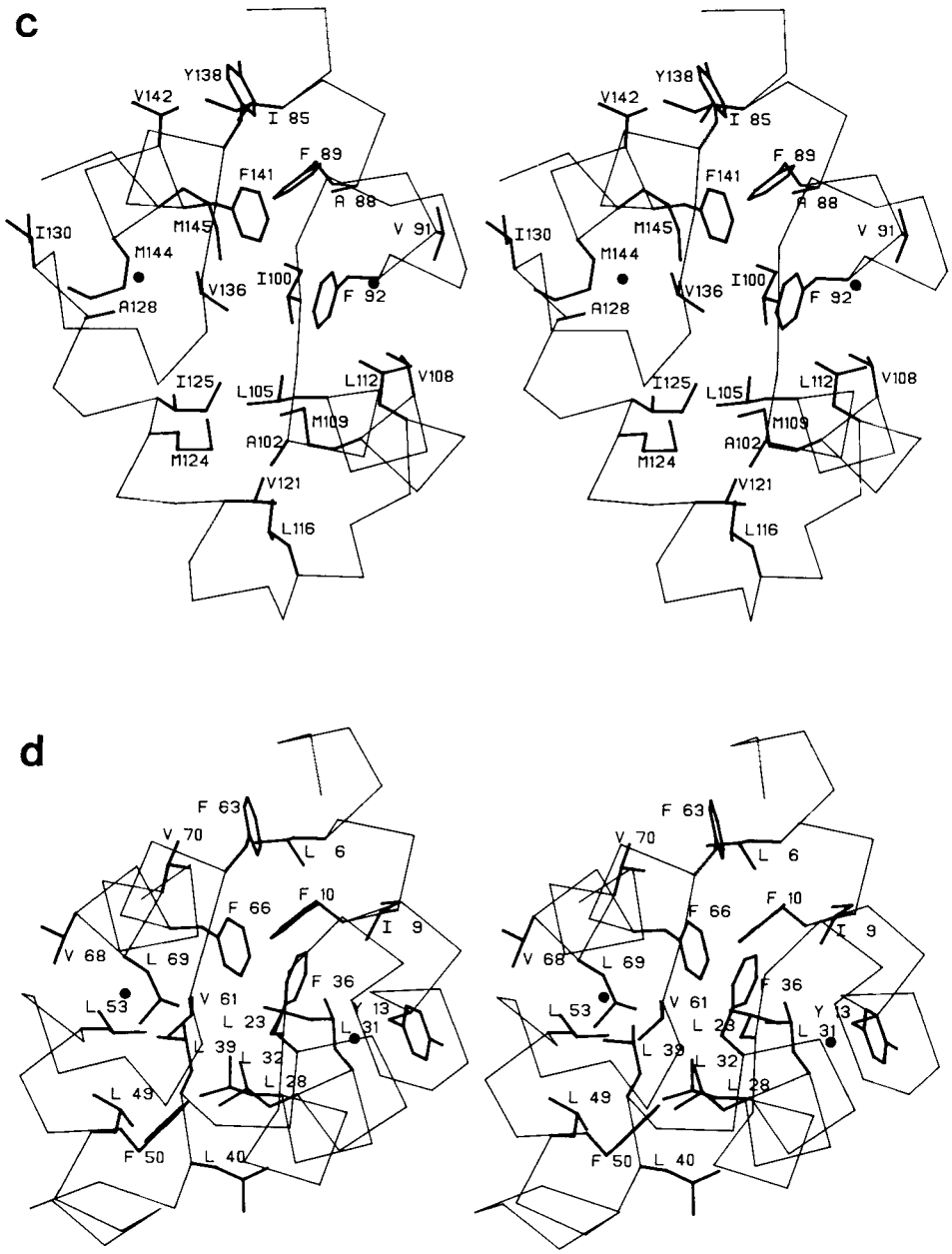

Fig. 3. c and d.

tary hydrophobic area on TnC or CaM with which to interact favorably. From the crystallographic structures[6–8] it appears that only the exposed hydrophobic pockets found in the calcium-bound domains could accommodate these helices.

The second property common to all the TnC/CaM inhibitory peptides is the presence of a cluster of residues that are positively charged at neutral pH (Table I). The importance of electrostatic charge interactions in peptide binding has been implicated in several previous studies. For example, the affinity of β-endorphin for CaM is significantly decreased as ionic strength is increased.[39] As well, amphiphilic peptide inhibitors designed with acidic instead of basic residues comprising their hydrophilic face failed to bind to CaM.[48]

Mastoporan, mastoporan X, and P. mastoporan constitute a family of naturally occurring tetradecapeptides isolated from the vespid wasp.[33] As mastoporan is considered to be one of the most potent peptide inhibitors of both TnC and CaM, it seemed a logical first choice for our modeling study.

The appropriate residues of mastoporan were aligned with the A-helix of TnC as shown in Table I.

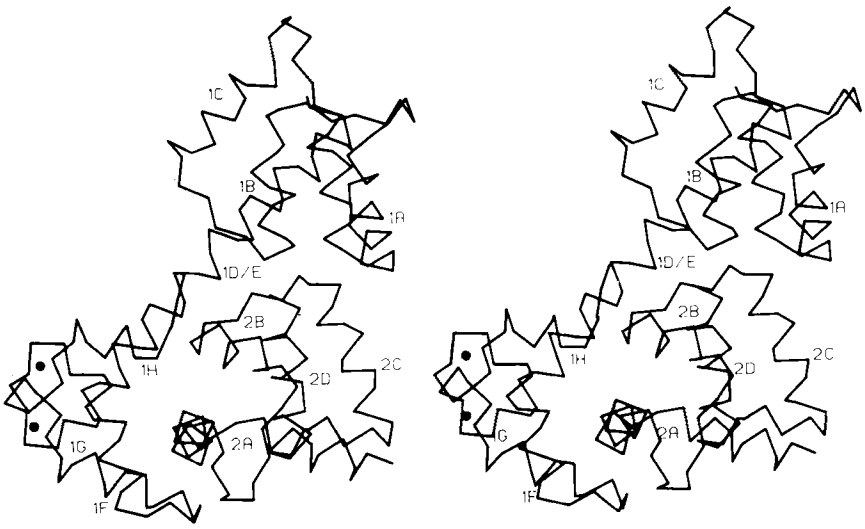

Fig. 4. A stereographic Cα tracing of the two symmetry-related TnC molecules depicting their interaction as observed in the X-ray crystallographic structures.[6,7] The helices of the two molecules are labelled 1A–1H, and 2A–2D/E (for nomenclature see reference 6). The C-terminal domain of molecule 1 is oriented as shown in Figure 2b, i.e., ~90° to the views in Figures 2a and 3. Calcium ions are shown as black-filled circles. The C-terminal domain of molecule 2 has been omitted to enhance clarity.

TABLE I. Alignment of TnC/CaM Inhibitory Peptides With the A-helix of TnC

A-helix	E16	E17	M18	I19	A20	E21	F22	K23	A24	A25	F26	D27	M28	—
Mastoporan	I1	N2	L3	K4	A5	L6	A7	A8	L9	A10	K11	K12	I13	L14
Mastoporan X	I1	N2	W3	K4	G5	I6	A7	A8	M9	A10	K11	K12	I13	L14
P. mastoporan	V1	D2	W3	K4	K5	I6	G7	Q8	H9	I10	L11	S12	V13	L14
Melittin	A4	V5	L6	K7	V8	L9	T10	T11	G12	L13	P14	A15	L16*	I17

*The full sequence of bee venom melittin is G1 I2 G3 [A4 V5 L6 K7 V8 L9 T10 T11 G12 L13 P14 A15 L16] I17 S18 W19 I20 K21 K22 K23 K24 Q25 Q26; brackets indicate the peptide region we have modeled onto TnC and CaM.

The modeled mastoporan provides a hydrophobic face to pack into the C-terminal hydrophobic cleft of TnC, with potential electrostatic interactions occurring along the outsides of the pocket. However, whereas the hydrophobic interactions in the A-helix/TnC case seem weak because they are from alanyl side chains predominantly, the potential hydrophobic interactions in the binding of mastoporan are more extensive and undoubtedly play a major role in the high affinity binding of this molecule for TnC. Figures 2c and 5b depict the structural fit of the mastoporan helix into the hydrophobic groove on the C-terminal domain of TnC, and Table II gives a summary of the proposed potential hydrophobic and electrostatic interactions.

Mastoporan provides a total of seven hydrophobic residues that could potentially interact with the hydrophobic residues in the cleft of the C-terminal domain. The positively charged amino terminus of the modeled drug is favourably close to the negatively charged cluster of residues, Glu133 and Asp136. Ile1 contacts Val129 as well as the aliphatic portions of the side chains of Glu127 and Glu133. Asn 2 is near Glu133 and Asp136, both of which protrude from the lip of the hydrophobic pocket. The Cβ of Asn2 is near the Cβ of Leu137. Leu3 forms a hydrophobic network with Leu137 and Val161 that extends from the H-helix of TnC. By conferring a continuous α-helical conformation upon helix H in TnC, Val 161 now provides potential extensive hydrophobic interactions with residues of mastoporan (Leu3, Leu6). It is interesting to note that a valine at position 161 is highly conserved among all the sequenced troponin C's[55–57]; the equivalent residue in CaM is Lys148. Lys4 extends to the outside of the mastoporan amphiphilic helix, where it is in a favorable position to form an electrostatic interaction with the side chain of Glu127 that extends from the F-G linker in TnC. The proposed alignment shown in Table I indicates a lysine at this position in the many known amphiphilic peptides that bind TnC and CaM as well as the highly conserved presence of a glutamic acid residue at position 127 in both TnC and CaM. Ala5 of mastoporan interacts with Val129 on the F-G linker peptide. Leu6 is a key residue in the mastoporan/TnC interaction as it forms favorable interactions with Met157, Met158, Leu137, and Val161 (Table II). Table I indicates that in the alignment proposed,

this position is invariably a hydrophobic residue, often a Leu or Ile in other amphiphilic inhibitory peptides. Ala7 and 8 both point to the outside of the helix. Ala7 is relatively exposed to solvent, whereas Ala8 is more protected with its Cβ pointing in at the Cβ of Thr125. Leu9 is another key contact residue with close hydrophobic contacts to Phe105 and Ile121. Ala10 interacts weakly with Met158 (4.0 Å). Mutation to a slightly larger side chain at this position might increase binding by filling in the small cavity around this alanine. Lys11 and Lys12 both extend into the solvent on the hydrophilic side of the mastoporan helix. The positively charged ammonium ion of lys12 effectively "caps" the F helix at its C-terminus, the most negative portion of its helix dipole. Ile13 binds to Ile104 and Phe105. Leu14, in turn, interacts favorably with the adjacent Ile13 in mastoporan itself.

Analysis of the Modeled Interaction of Mastoporan With the C-Terminal Domain of Calmodulin

The extensive sequence and structural homology of TnC and CaM is well documented and was the basis of our earlier computer modeling of CaM based on the structure of turkey skeleton TnC.[19] Superpositioning of the Cα atoms of the C-termini of TnC and CaM yields a rms of less than 1.0 Å.[9] This fact, in combination with the high degree of conservation of hydrophobic residues within the hydrophobic pockets of TnC and CaM (compare Fig. 3a and c) would suggest that mastoporan could bind in similar positions in the two molecules. Residues involved in our model of mastoporan binding to CaM (Fig. 5d) include the following (the analogous residue in TnC is in brackets): Ala88 (Cys101), Val91 (Ile104), Phe92 (Phe105), Val108 (Ile121), Met109 (Leu122), Leu112 (Thr125), Glu114 (Glu127), Glu123 (Asp136), Met124 (Leu137), Met144 (Met157), and Met145 (Met158). In addition, the alkyl side chain of Lys148 (Val161) could interact with Leu3 of mastoporan. The replacement of an Ala for a Cys at position 88, of a Met for a Leu at position 109, and of a Leu for a Thr at position 112 serves to increase the hydrophobic surface area in CaM with which mastoporan may interact.

The smaller interhelical angles of the HLH unit in calmodulin (as compared to TnC[9]) serves to promote closer fit of the amphiphilic helices into the hydrophobic patch. This is mainly due to the altered position of the loop joining the two helices (compare Fig. 5b and d). This phenomenon, in addition to the increased hydrophobic interaction area mentioned above, may contribute to mastoporan's greater association with calmodulin in comparison to TnC.

A differential trace labelling study of lysine residues in CaM showed that binding of either an amphiphilic inhibitory peptide (β-endorphin) or the antipsychotic drug TFP decreased the reactivities of Lys75 and Lys148 most dramatically,[58] thus suggesting common binding sites for the two molecules. Lys75 extends across the N-terminal hydrophobic domain; Lys148, although not seen in the crystal structure[8] is thought to lie in an analogous manner near the C-terminal hydrophobic domain.[19] Indeed, several of the residues in CaM proposed to be involved in binding mastoporan are also those proposed to be involved in the binding of TFP to CaM.[19] This observation would help explain the competitive effect of TFP on the binding of these amphiphilic helices to CaM.[13,19,38,39] Furthermore, as discussed in the earlier TFP modeling study, several of these hydrophobic residues on CaM as well as Lys75 and Lys148 would be substantially buried in the Ca^{2+}-filled (open) to Ca^{2+}-free (closed) transition.[19] Therefore, as with the case of TFP binding, one would also expect a decreased affinity of the modeled amphiphilic helical mastoporan peptide for CaM as the accessible hydrophobic surface with which it could interact is severely diminished in the Ca^{2+}-free state. This would explain the calcium requirement for the high affinity interaction of the amphiphilic helices to CaM and TnC.

Binding of Amphiphilic Helices to the N-Terminal Domain of CaM

Although different experimental conditions have led to conflicting reports, there is evidence that 2 moles of mastoporan[59,60] are bound per mole CaM; one with high affinity and one with lower affinity. A ^{113}Cd NMR study found that 2 moles of mastoporan/mole of CaM broadened the four ^{113}Cd NMR signals over that found in a 1:1 molar ratio without affecting the chemical shifts.[59] This observation was interpreted as binding of a second molecule of mastoporan with lower affinity than the first; the absence of changes in the ^{113}Cd NMR chemical shifts argues against major conformational changes in calmodulin (at least near the metal binding sites), as the second peptide molecule binds to the protein. Proteolytic digestion studies of CaM showed that the C-terminal 78–148 fragment bound mastoporan with an affinity equal to that of the intact protein.[60] The N-terminal 1–77 fragment also bound mastoporan, but with reduced affinity (K_a 10^5 M^{-1}). This reduced affinity may be due to disruption of the D-helix. Indeed, it had been shown earlier that the 1–106 fragment of CaM bound mastoporan with higher affinity.[38] This example highlights the inherent limitations of tryptic digestion studies in defining molecular binding sites. The choice of fragmentation is often limited, and may give negative results simply because the tertiary structure of the binding area is being destabilized by the removal of adjacent secondary structural units. For instance, the 107–148 tryptic fragment of CaM was found to bind very weakly to mastoporan, and the area was subsequently dismissed as not being the major site for

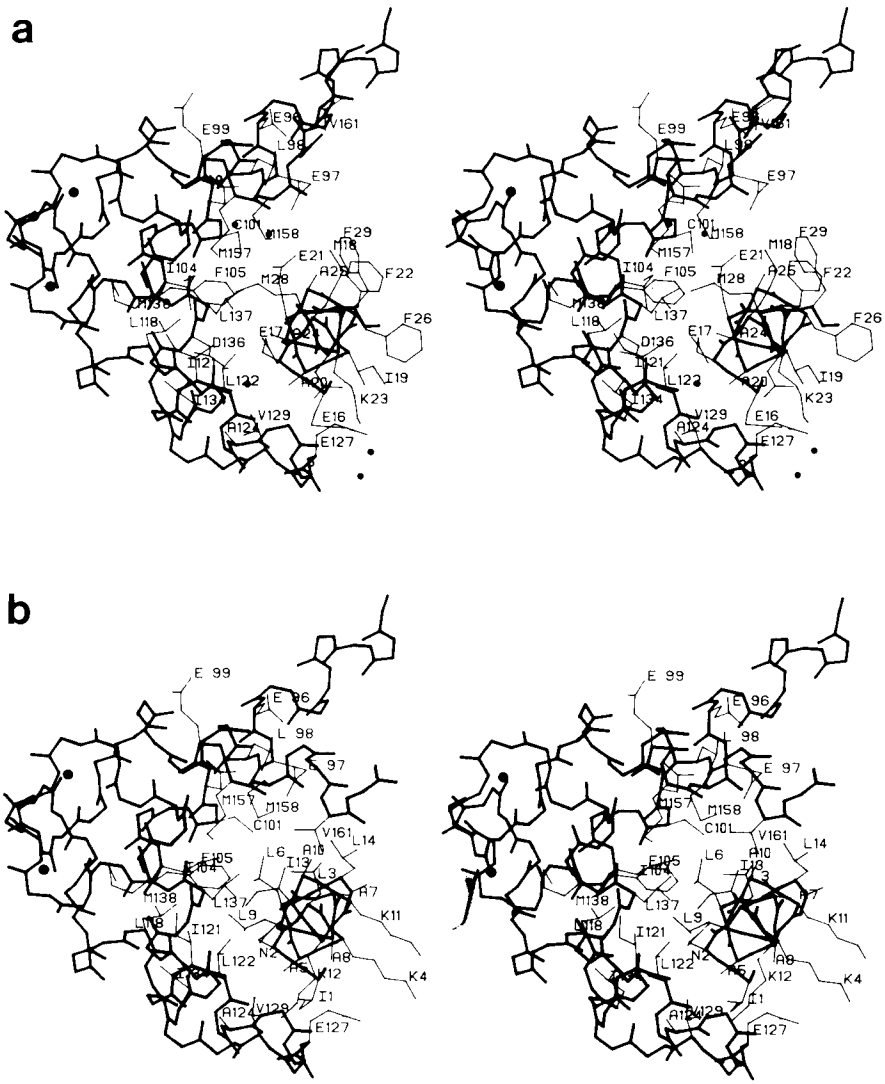

Fig. 5. The interactions of the C-terminal domain of TnC and CaM with amphiphilic helices: main chain is in dark lines, side chains in light. Calciums are black circles, waters are smaller black circles. **a:** The crystallographically observed interaction of TnC with the A-helix of its symmetry related neighbor. **b:** The modeled interaction of mastoporan with TnC. **c:** The modeled interaction of a segment of melittin with TnC. **d:** The modeled interaction of mastoporan with the C-terminal domain of CaM.

drug binding.[38] However, perusal of the CaM structure shows that removal of amino acids 85–106 (which includes all of Ca^{2+}-binding loop III and several key residues of the domain's hydrophobic core[9] would be devastating to the conformation of the C-terminal domain.

That mastoporan would bind simultaneously to both the N- and C-terminal domains of CaM might be expected considering the extensive homology between the two domains, especially in the hydrophobic pocket regions, where we propose that the peptides will bind (compare Fig. 3b and c). Mastoporan is small enough that binding to one domain would not interfere with binding at the second. All of the hydrophobic residues of CaM involved in peptide complex formation in the C-terminal domain have analogous counterparts in the N-terminal domain. A notable difference in the N-terminal binding site is a smaller number of negatively charged side chains surrounding the hydrophobic pocket. Most significantly, there is no counterpart to Glu114 of the C-terminal domain, which is in a favorable position to interact with the lysine extending off the mastoporan helix at position 4. This in part may account for the lower affinity of peptide for the N-terminal domain of CaM.[60]

Recently, a computer molecular-modeling study involving calmodulin and its interactions with the

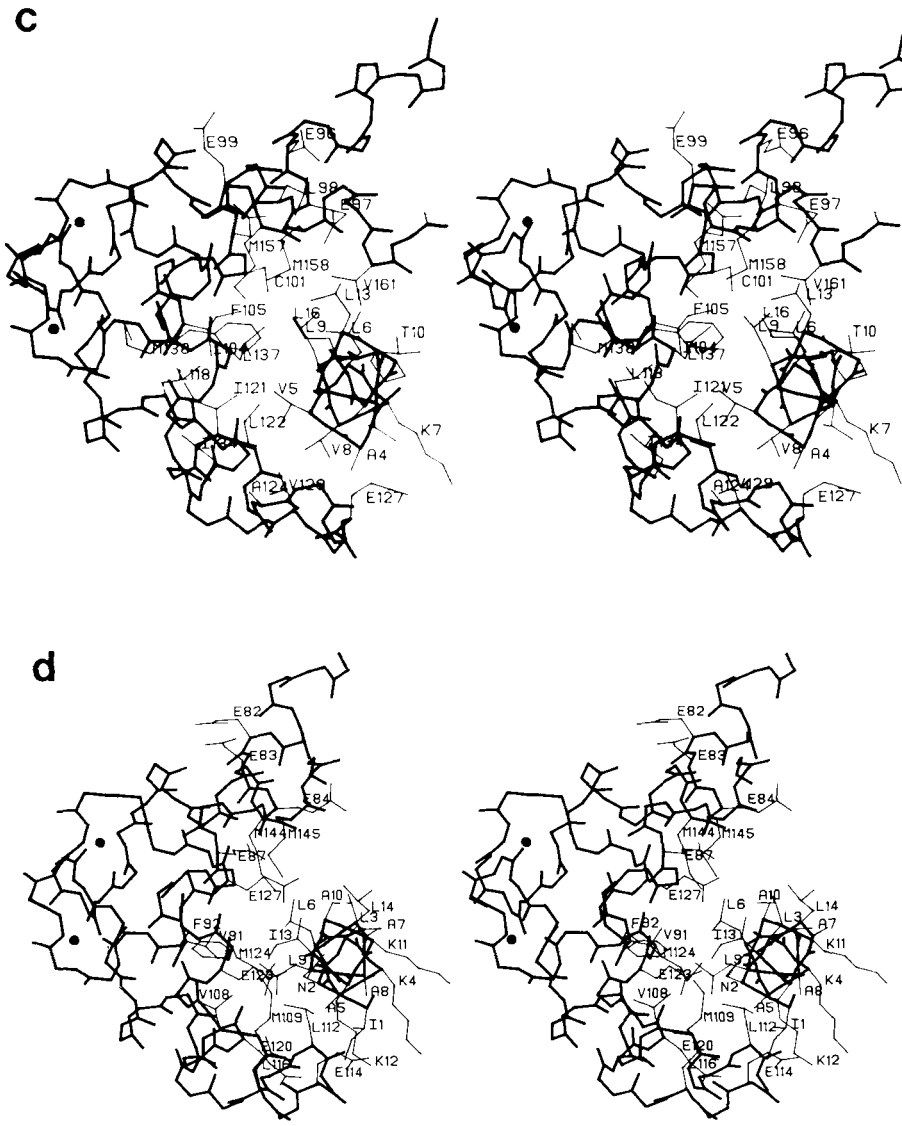

Fig. 5. c and d.

calmodulin-binding domain peptide from MLCK has been described.[61] In that model, the 27 amino acid peptide was given an α-helical conformation and the authors proposed that it interacts simultaneously with both the N-terminal and C-terminal hydrophobic patches of CaM. This work was a logical extension of the earlier cross-linking study[62] that suggested the central helix of CaM could act as a "flexible tether" to accommodate interactions with a variety of target enzymes. Subsequently, support for this modeling has come from a series of deletion mutants of CaM that retain their activation ability for some targets in spite of the removal of segments of the central helix.[63]

In order to accomplish the MLCK peptide-CaM complex model building, the authors introduced a non-helical bend ($\phi = -54$, $\psi = +98$) into the central linker helix of CaM at Ser81. This single change was not sufficient to achieve the required interactions, so smaller adjustments were made to the main-chain torsional angles of several residues adjacent to Ser81. The resulting proposed conformational change brings the hydrophobic patch on each domain to face towards one another so that hydrophobic residues on one side of the MLCK peptide would interact with the N-terminal domain and at the same time other hydrophobic residues on the opposite side of the peptide would interact with the C-terminal domain.

In its present form, we found that the bent helix model[61] could not account for simultaneous interactions of residues on a single helical peptide with the

TABLE II. Summary of the Interactions of the Observed and Modeled Amphiphilic Helices With TnC*

TnC A-helix												
E16	E17	M18	I19	A20	E21	F22	K23	A24	A25	F26	D27	M28
E127	D133			L122	O261			F105				I109
O197				V129				L122				F105
				E127				I121				
Mastoporan												
I1	N2	L3	K4	A5	L6	A7	A8	L9	A10	K11	K12	I13
V129					L137							
E133	E133	L137	E127	V129	M152			F105	M158			I104
D136	D136	V161		E127	M158		T125					F105
	D137				V161			I121				
Melittin												
A4	V5	L6	K7	V8	L9	T10	T11	G12	L13	P14	A15	L16
				L122	L137				M158			I104
	L137	L137	E127	V129	M157				F105			F105
	E133	V161		E127	M158							
	D136				V161							

*Residues of the helices are given across the page in rows. Residues of TnC with which they interact are given below them in the columns. All distances are acceptable hydrophobic contacts (less than 3.8 Å) except for a few long contacts (4.4 Å or less) to alanine side chains.

hydrophobic regions of both domains of CaM. The average distance between hydrophobic residues on the opposing N- and C-terminal patches is approximately 20 Å, a distance far too large to allow for effective hydrophobic interactions (~3.8–4.2 Å) from both domains to the same helical peptide. In addition to this disparity in the interdomain distances there are several other reasons why such a model can not easily reconcile the high affinity binding of mastoporan to CaM.

First, as mentioned above, the isolated C-terminal tryptic peptide of CaM has an affinity for mastoporan that is almost equal to that of intact CaM.[60] The bent-helix model would predict a quite different affinity between these two forms of CaM which is clearly not the case. Second, even if it were possible to bring the two domains sufficiently close together so they could interact simultaneously with a single peptide, the strongly amphiphilic character of mastoporan would require one of the domains to interact with a predominantly polar/charged surface of the helical peptide. Certainly charged residues such as lysines can contribute to hydrophobic interactions via the aliphatic portion of their side chains, but their ionic moieties must still somehow be satisfied in the apolar environment. Table I indicates that the charged face of mastoporan would present three such moieties (Lys4, Lys11, Lys12) to one of the hydrophobic domains of CaM in the bent helix model. In light of the similarity of the N- and C-terminal hydrophobic domains of CaM, and the small size of mastoporan, it is difficult to reconcile why it would be more energetically favorable to distort the central helix of CaM so that one of the hydrophobic domains would interact with a charged face of a single peptide rather than to bind the hydrophobic face of a second peptide molecule.

A Model for the Interaction of Melittin Binding to CaM

Like mastoporan, bee venom melittin binds with high affinity (10^9 M^{-1}) to CaM and contains a 14 residue segment with an amphiphilic, helical nature.[34,41,42,44,52,53,64,65] However, melittin differs from mastoporan in that this 14 residue segment is embedded within a larger 26 residue peptide (Table I). Additionally melittin binds CaM with a 1:1 stoichiometry. Binding of 1 mole of melittin to 1 mole of CaM affects residues in both the N- and C-terminal domains.[52] Finally, melittin can complex with both the 1–77 and the 78–148 tryptic fragments but with reduced affinity for both (.5 μM and 2 μM, respectively.[66]

The sequence alignment of a 14 residue amphiphilic segment of melittin to the A helix of TnC and the mastoporans is given in Table I. In this alignment, the key TnC/CaM binding residues of mastoporan, Leu3, Lys4, Leu6, Leu9, are also retained in melittin (Leu6, Lys7, Leu9, and Leu13). Indeed we were able to construct a preliminary model of this 14 residue section of melittin complexed with TnC and CaM which is strikingly similar to that of mastoporan (see Table II; Fig. 5c). As for mastoporan we would predict that the hydrophobic face of this 14 residue segment of melittin could interact with analogous hydrophobic residues in either the N- or C-terminal domains of CaM.

Although the binding of this 14 residue portion of melittin seems reasonable, especially in light of its

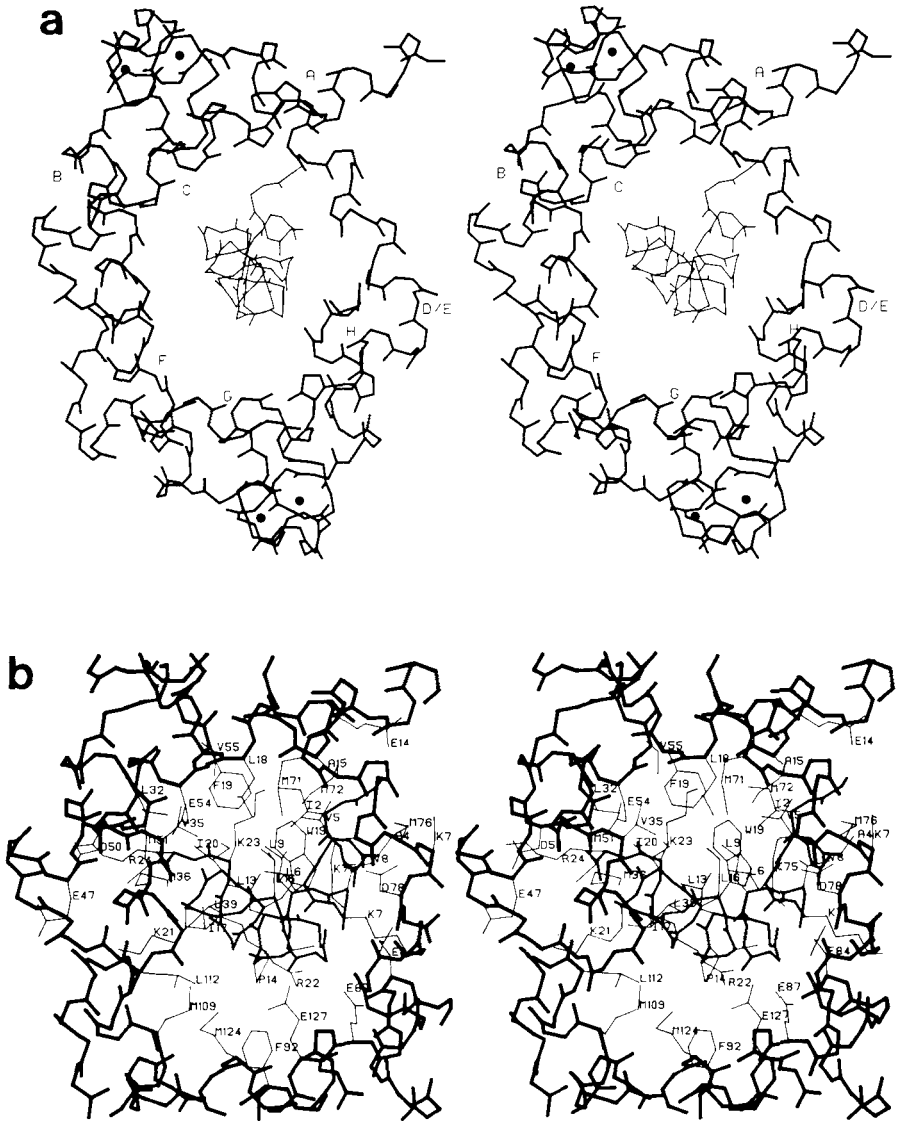

Fig. 6. **a:** A stereographic representation of the modeled interaction of melittin with bovine brain calmodulin. For clarity, only main chain atoms are represented. CaM is in thick lines, melittin in thin lines. Calciums are black circles. Helices of CaM are labeled A–H. **b:** A stereographic view depicting side chain interactions within the melittin/CaM model. CaM and melittin main chain are in thick lines; side chains in thin lines. The orientation of the figure is identical to that shown in Figure 5a, only enlarged.

strong homology with the mastoporan/CaM case, our model at this point did not account for the additional C-terminal ten residues on melittin.

Judging from the cluster of positively charged basic residues in the C-terminus of melittin and the proximity of a cluster of negatively charged acidic residues on the D/E linker of CaM and TnC, it seemed that this would be a favorable interaction site. Fluorescence labeling and tryptic digestion studies have also implicated the D/E linker region as important in binding melittin.[38,64,66] If the position of the amphiphilic 14-residue segment of melittin in the hydrophobic cleft is to be maintained, then it is obvious that a bend in the melittin molecule would be required to redirect the positively charged C-terminal portion up along the D/E helical linker. Flexibility of the main chain dihedrals of Gly12 and Pro14 in melittin could favor such a bend. Indeed, a kinked conformation in the melittin helix was seen in the X-ray crystallographic structure solution of the isolated molecule.[53]

With this in mind, we then proceeded to model the full melittin molecule onto CaM. What was immediately evident, however, was that to maintain hydrophobic interactions of melittin with the C-terminal apolar cleft and to accommodate interactions of the C-terminal tail with the D/E helix of CaM required a stereochemically unreasonable bend at

Gly12/Pro14 of melittin. We found that the only way to position a more reasonably curved melittin helix was to rotate the N-terminal segment of melittin by 180° around its helix axis. This movement allows the dipole of the C-terminal helix of melittin to run in a favorable manner relative to the D/E helix of CaM, and allows contact of oppositely charged residues on the two molecules. However, this rotation also served to place the partly hydrophilic face of the N-terminal amphiphilic segment of melittin into the C-terminal hydrophobic cleft of CaM, and conversely moves its hydrophobic face into an exposed state. We predict that this apparently unfavorable position for melittin may be accommodated by a modified version of the Kretsinger bent helix model.

Table I shows that the polar face of the 14 residue amphiphilic segment of melittin is much less charged than mastoporan, containing only one positively charged lysine residue. Lys11 and Lys12 of mastoporan are substituted by Pro14 and Ala15 in melittin, both small non-polar residues. The only other polar residues on the hydrophilic face are Thr10 and Thr11. Such small neutral polar residues are more amenable to burial at molecular interfaces because they can form satisfying hydrogen bonds with main chain carbonyl oxygen atoms in α-helices.[67] Therefore, the movement of the polar melittin face into the C-terminal hydrophobic cleft may be reasonable.

Provided that the central helix of CaM could be distorted sufficiently, the exposed hydrophobic face of melittin could then be satisfied by interactions with apolar residues in the N-terminal hydrophobic pocket of CaM. By altering the original concept of Persechini and Kretsinger,[61] we have modeled a more severe kink of the central helix of CaM. The largest change again involved the conformation at Ser81 ($\phi = -85$, $\psi = 98$). This alteration in torsion angles serves to bring the two hydrophobic domains of CaM sufficiently close to bind a single peptide simultaneously while still maintaining reasonable stereochemistry and hydrogen bonding patterns within the kinked central helix (see Fig. 6a). This mode of interaction would predict a more compact size for the CaM/melittin complex (~46 Å) than in native CaM (~65 Å) or that indicated in the Kretsinger CaM/MLCK model (~50 Å). Our model would also explain the 1:1 stoichiometry of the melittin/CaM complex, and the apparent involvement of both CaM domains in the binding of a single melittin peptide.[52]

Detailed hydrophobic interactions of the CaM/melittin complex are shown in Figure 6b. The majority of favorable nonpolar contacts involves residues in the N-terminal hydrophobic cleft of CaM; the primary residues involved (Phe19, Leu18, Leu36, Met51, Met71, Met72) all have analogous counterparts in the C-terminal domain and are the same residues implicated in the binding of mastoporan. Val5, Leu6, Val8, Leu9, Leu13, and Ile20 of melittin form the significant hydrophobic face which binds in the CaM N-terminal hydrophobic cleft.

There are also several electrostatic interactions in the proposed melittin/CaM complex. Glu14 (CaM) is in a favorable position to interact with the positively charged N-terminus of melittin. Lys7 could form a favorable ionic interaction with Glu127. Lys21 is found lying near the carboxy terminus of helix F. Positively charged residues are often found at the C-terminal ends of helices where they interact with the negative component of the helix dipole.[68] Arg22 could interact electrostatically with Asp78, Glu84, and Glu87. Lys23 caps the carboxy end of helix C. Arg24 is proximal to a cluster of acidic residues Glu47, Asp50, and Asp54 on helix C.

In addition to their role in charge-charge interactions, the aliphatic portions of Asp, Glu, Lys, and Arg residues in CaM also serve to provide many hydrophobic interactions with melittin. For example, Arg74 and Lys75 of CaM surround and effectively bury the Trp side chain with the nonpolar portion of their side chains. This could explain the dramatic loss of fluorescence of Trp19 of melittin upon binding to CaM.[66]

CONCLUSIONS

The main purpose of this study was to understand how amphiphilic helical molecules might associate with the calcium-binding regulatory proteins TnC and CaM. The experimental observation of a relatively favorable binding interaction of an α-helical segment into the C-terminal domain hydrophobic cleft (as seen in the high resolution picture of the TnC crystal lattice) was used as a guideline to model such a binding site. The modeled amphiphilic helices could be fit into an analogous position in the apolar cleft, forming several favorable molecular interactions without sacrificing standard stereochemical constraints. Their positions and interactions with various residues in the hydrophobic clefts which are exposed only in the Ca^{2+} bound state[11,19] would account for their Ca^{2+}-dependent affinity for CaM and TnC. In the Ca^{2+}-free form, the hydrophobic cleft would be substantially buried.

The interaction of extracellular peptides such as mastoporan and melittin with intracellular proteins such as TnC and CaM is not physiologically relevant. However, because several of the binding sites for CaM on target molecules are thought to be relatively short amphiphilic helical regions our model may help in understanding the nature of these interactions.

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