Thermodynamics of target peptide recognition by calmodulin and a calmodulin analogue: implications for the role of the central linker

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Abstract The thermodynamics of interaction of two model peptides melittin and mastoparan with bovine brain calmodulin (CAM) and a smaller CAM analogue, a calcium binding protein from *Entamoeba histolytica* (CaBP) in 10 mM MOPS buffer (pH 7.0) was examined using isothermal titration calorimetry (ITC). These data show that CAM binds to both the peptides and the enthalpy of binding is endothermic for melittin and exothermic for mastoparan at 25°C. CaBP binds to the longer peptide melittin, but does not bind to mastoparan, the binding enthalpy being endothermic in nature. Concurrently, we also observe a larger increase in α-helicity upon the binding of melittin to CAM when compared to CaBP. The role of hydrophobic interactions in the binding process has also been examined using 8-anilino-1-naphthalene-sulphonic acid (ANS) binding monitored by ITC. These results have been employed to rationalize the energetic consequences of the binding reaction.

Key words: Calmodulin; Calcium binding protein; Isothermal titration calorimetry; Melittin; Mastoparan

1. Introduction

Intracellular calcium plays an essential role in signal transduction in several biological systems. A key element in this process seems to be the conformational change induced in the target proteins upon metal ion binding. These conformational changes appear to be critical for the binding of these proteins to their effector molecules and the subsequent induction of function. An archetypal example of this class of calcium binding proteins is the small, two domain protein calmodulin (CAM) [1]. The ubiquitous nature of CAM lends credence to its role as a trigger in the calcium signaling process. This dumbbell-shaped protein has two domains, each having two calcium binding sites. These two domains are connected by a long central linker, a helix in the crystal structure [2–4] but which is flexible in solution [5,6]. Crystal structures of CAM-target peptide complexes reveal a reorientation of the two domains following a bend in the central helix upon complexation [7]. Apart from binding to target peptide stretches, the linker has also been proposed to modulate metal ion binding and inter-domain cooperativity [8]. These reports need to be examined in the light of earlier reports which suggested the independence of the two calcium binding entities and also studies which postulate a role for the electrostatic potential in the coupling between the two domains [9].

The present paper reports studies aimed at elucidating the role of the central linker in a CAM-like system, a calcium binding protein (CaBP) from *Entamoeba histolytica* [10–12], by comparing its target recognition features with that of the archetypal CAM. This CaBP (134 residues) is smaller than CAM and has a two residue insertion in the central linker (Fig. 1A). The central linker in this case contains three glycines, a feature exhibited by CAMs and CAM analogues/homologues in most plants and lower eukaryotes [13].

The present studies were designed to quantitatively analyze the energetics of the interaction of CAM and CaBP with the model peptides melittin (the 26 residue peptide from bee venom) and mastoparan (a 14 residue peptide) (Fig. 1B). The thermodynamic parameters of the binding of these peptides were obtained using isothermal titration calorimetry (ITC). Spectroscopic evidence towards the induction of secondary structure in the model peptides, which are otherwise unstructured in solution at neutral pH, was also examined in the context of properties reported in literature [14]. In parallel, we have also tried to rationalize the data on the binding of these model peptides in terms of estimates of the binding of 8-anilino-1-naphthalene-sulphonic acid (ANS) and used an indicator for the role of the central linker in modulating these interactions. Molecular models to visualize the domain movements resulting from an extended linker in CaBP have also been constructed and compared with that of CAM.

2. Materials and methods

2.1. Materials and purification

The plasmid encoding the gene for CaBP was expressed in the *Escherichia coli* strain BL21(DE3)pLysS. The purification protocol used was as previously described [15]. Bovine brain CAM was prepared by the method of Gopalakrishna et al. [16]. The purity of both proteins was checked by sodium dodecyl sulfate gel electrophoresis. Protein concentration was estimated using the molar extinction coefficient (ε_{280}) of 5120 M^{-1} cm^{-1} for CaBP and 3300 M^{-1} cm^{-1} for bovine brain CAM. *Apo* CaBP and *apo* CAM were prepared by procedures described earlier [11]. Plasticware was used throughout to avoid metal ion contamination. Melittin and mastoparan were purchased from Sigma and purified using reverse phase HPLC. The concentration of peptide stock solutions were determined by ε_{280} of 5600 M^{-1} cm^{-1} for both the peptides. ANS was also purchased from Sigma and its concentration determined by ε_{350} of 5000 M^{-1} cm^{-1}.

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Abbreviations: CAM, calmodulin; CaBP, calcium binding protein; ITC, isothermal titration calorimetry; ANS, 8-anilino-1-naphthalene-sulphonic acid; MOPS, 3-[*N*-morpholino]propane-sulphonic acid; smMLCK, smooth muscle-myosin light chain kinase

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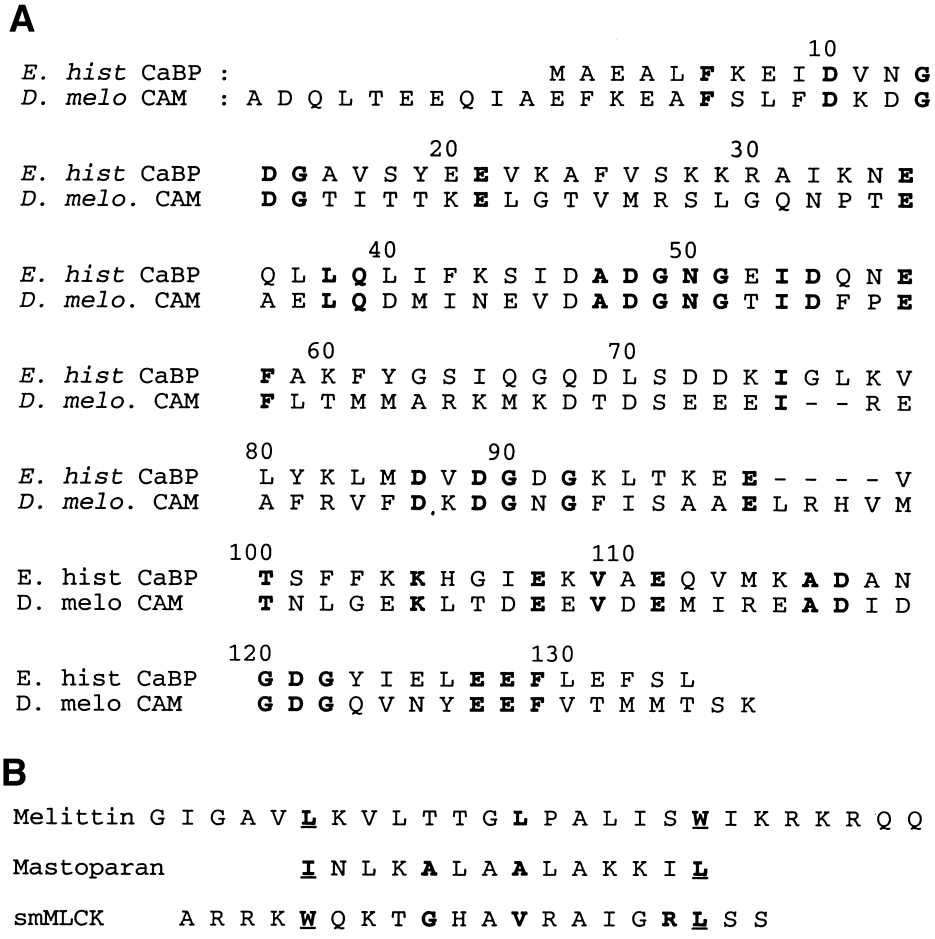

Fig. 1. A: Sequence comparison of CaBP with mammalian recombinant CAM. Note the insertion of the two extra residues in the linker region. B: Sequence alignment of the prototype CAM binding fragment models (adapted from [18]). The putatively conserved major (bold and underlined) and minor (bold) hydrophobic anchors are also indicated.

2.2. Circular dichroism spectroscopy

Circular dichroism (CD) studies were carried out on a Jasco J500 spectropolarimeter attached to a DP-501N data processor. Far-UV Spectra were collected at a scan speed of 10 nm/min and a response time of 8 s. Each spectrum was obtained as an average of four scans. The wavelength range scanned was 200–250 nm, with a typical protein concentration of 20 μM and peptide concentration of 5 μM using a cuvette with a path length of 0.1 cm. All the samples were prepared in 10 mM 3-[*N*-morpholino]propane-sulphonic acid (MOPS) buffer (pH 7.0). A Julabo circulating water bath was used to maintain the samples at a constant temperature.

2.3. Isothermal titration calorimetry

Titration calorimetry studies were carried out using a Microcal Omega titration calorimeter [17]. A typical titration consisted of injecting 2.4 μl aliquots of 1.5 mM peptide solution into 0.1 mM of holo CaBP/CAM in 10 mM MOPS buffer (pH 7.0). For the ANS binding studies, a 2.1–3.2 μl aliquot of 20 mM ANS was injected into 0.1 mM of *holo* CaBP in 10 mM MOPS buffer (pH 7.0) with and without peptide and heat changes monitored.

3. Results

The change in the far-UV CD spectra upon binding of melittin are more pronounced in the case of CAM as compared to CaBP (Fig. 2.). ITC data shows that the *apo* forms of these proteins do not bind to the peptides studied (data not shown). Typical titrations for the binding of CAM to melittin and mastoparan are shown in Fig. 3. which demonstrate that reactions of the former are endothermic in nature while that of the latter are exothermic. CaBP-melittin interactions are also endothermic while mastoparan fails to bind to CaBP under identical conditions. The binding constants and associ-

Table 1
Thermodynamic parameters of peptide binding to CAM and CaBP

System	Stoichiometry	T (°C)	K_b (M^{-1})	ΔH (J/mol)	ΔC_p (J/mol K)
CAM-melittin	1.130	12	9.9E7 ± 1.3E5	2.98E4 ± 1.6E2	−1.28E3 ± 8.84E2
	1.040	25	7.1E7 ± 1E5	1.32E4 ± 8.7E2	
CAM-mastoparan	1.090	8	1.1E7 ± 1.3E5	−1.2E4 ± 2.4E2	−9.52E2 ± 2.0E3
	0.970	25	4.5E6 ± 1.5E5	−2.8E4 ± 2.0E3	
CaBP-melittin	0.980	12	9.1E5 ± 1.2E4	2.93E4 ± 3.2E3	−2.32E3 ± 4.8E3
	0.930	20	1.4E5 ± 1E4	1.08E4 ± 2.7E3	

ated thermodynamic parameters obtained from these experiments are reported in Table 1.

The isothermal titrations performed on calcium saturated CAM/CaBP to monitor the binding of ANS to these systems shows the presence of at least four sites for CaBP and 10 sites for CAM. ANS binding experiments performed using the protein samples bound to the peptides reveal that the CAM-melittin complex leaves very little hydrophobic surface exposed (only five molecules of ANS bind per complex molecule). On the other hand, the CAM-mastoparan complex exhibits a higher ability to bind to ANS with nearly eight molecules binding per complex molecule, thereby indicating a considerable exposure of the hydrophobic surface in the former. The CaBP-melittin complex, also, seems to lose a portion of its hydrophobic surface as seen by its reduced ANS binding capacity (2.5 molecules per complex molecule) when compared to native CaBP.

Models of the structure of CaBP-peptide complex were generated using the known crystal structure of CAM [7] bound to the smooth muscle-myosin light chain kinase fragment (smMLCK), to help visualize the structural features that could account for the observed differences in the spectroscopic and thermodynamic features of peptide binding to CAM and CaBP. The structure of the CAM-smMLCK complex showed the linker region (extending from residue number 66 to 91) to be segmented into two short stretches of α-helices, from 66–78 and 79–91 (in the NMR structure of skeletal muscle light chain kinase with CAM [6] the stretch of residues 74–82 are reported to form a large flexible loop). Sequence comparisons reveal the two-residue insertion in the

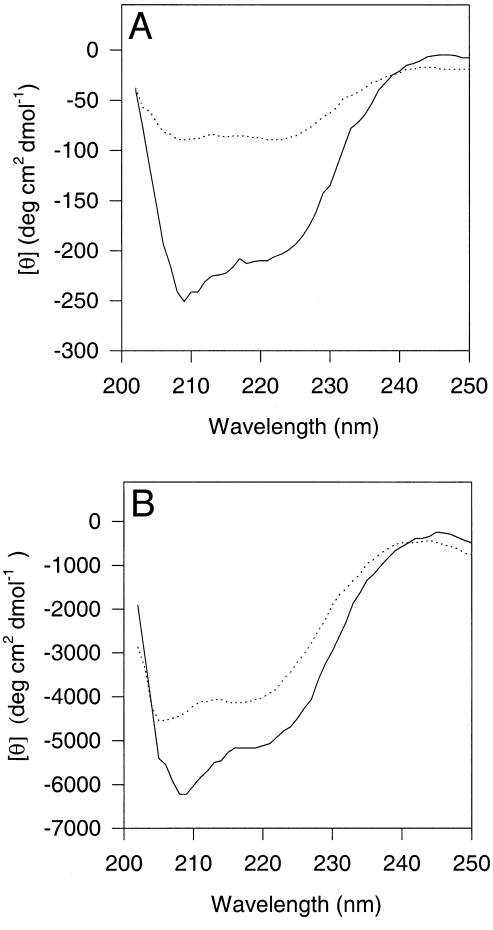

Fig. 2. Changes in the far-UV CD spectra upon melittin binding. (...) protein only and (—) protein in the presence of melittin. A: CAM and B: CaBP.

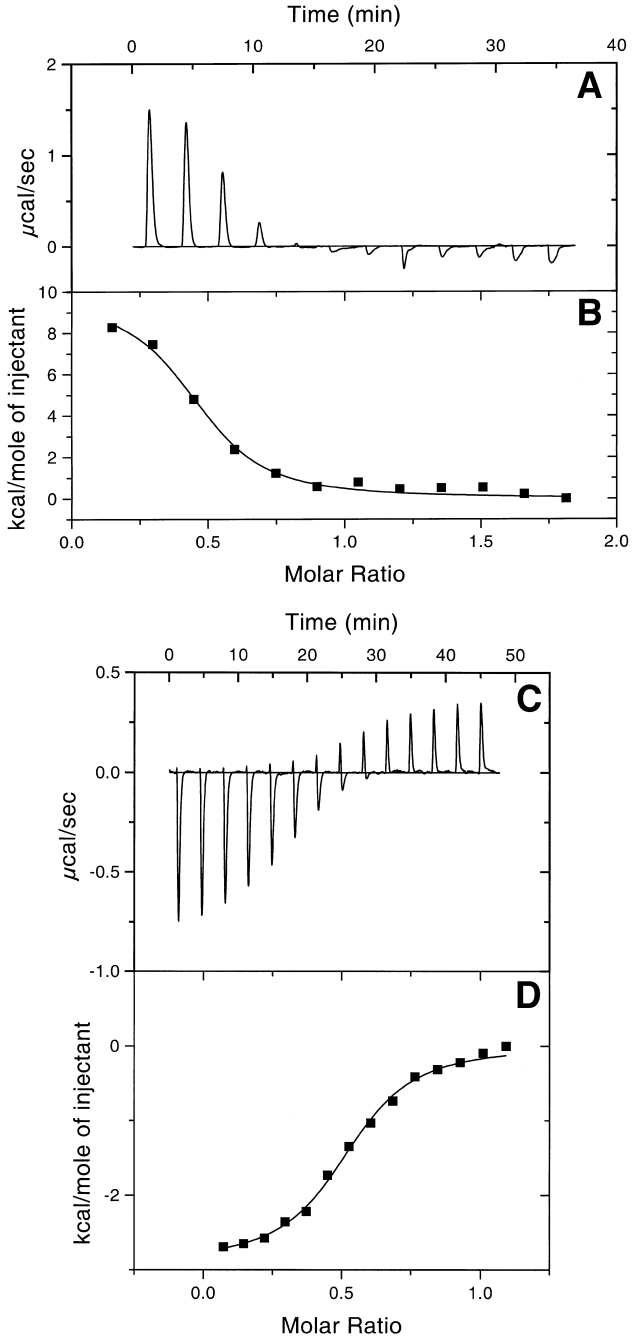

Fig. 3. A: A calorimetric titration profile of 2.4 μl aliquots of 1.5 mM melittin into 0.1 mM of holo CAM in 10 mM MOPS buffer (pH 7.0) at 25°C; B: A least squares fit of the data to the heat absorbed per mol of titrant versus the ratio of the total concentration of ligand to the total concentration of protein. C: A calorimetric titration profile of 2.4 μl aliquots of 1.5 mM mastoparan into 0.1 mM of holo CAM in 10 mM MOPS buffer (pH 7.0) at 8°C; D: A least squares fit of the data to the heat absorbed per mol of titrant versus the ratio of the total concentration of ligand to the total concentration of protein.

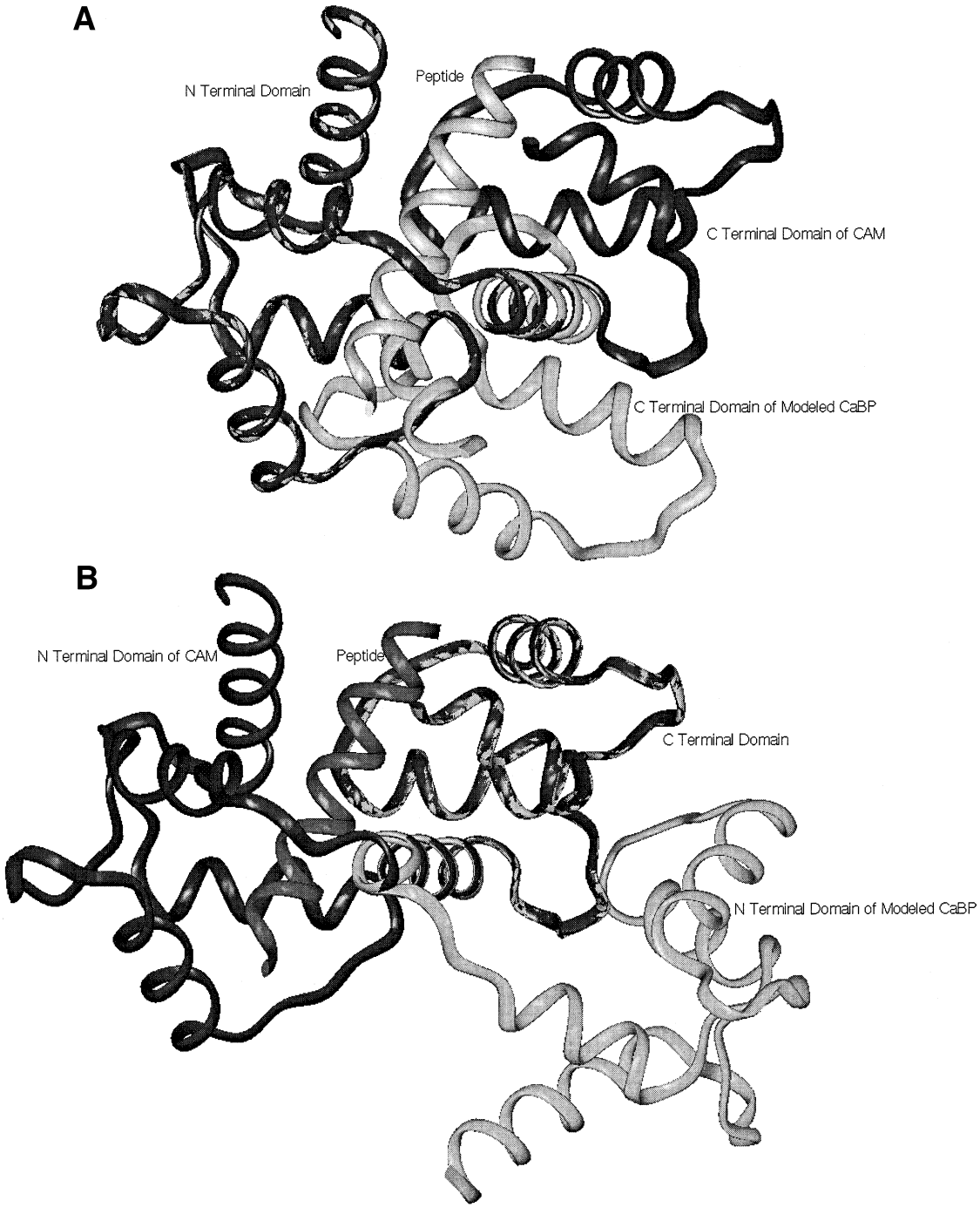

Fig. 4. CAM-smMLCK complex structure with the proposed CaBP model. The dark ribbon is CAM while the light one represents modeled CaBP. A: N terminal retained and C terminal reoriented. B: N terminal reoriented and C terminal retained.

linker segment of CaBP to occur in the second helix in the complex structure. The two residues were thus accommodated as part of the linker ∝-helix (Fig. 4A) which leads to the movement of the C terminal domain away from the peptide binding region thereby resulting in the reduction of the number of binding contacts between the peptide and the domain. This feature could plausibly represent the maximal deviation that the carboxy terminal domain can adopt upon peptide binding. In the second model the two-residue insertion is accommodated in the N terminal domain leading to the deviation of this domain from the bound peptide as shown in Fig. 4B.

4. Discussion

The specificity of target recognition by CAM has been reviewed [18]. The correlation between the target peptide sequence and its binding specificity/affinity to CAM has led to a debate on the nature of the interaction between CAM and the target peptides [19,20]. A number of elegant experiments

using a variety of techniques demonstrate the induction of ∝-helicity in the target peptides. Fluorescence studies [21], CD measurements [14], mass-spectrometry [22], small-angle X-ray scattering measurements [23], NMR structures [6] and crystallographic data [7] have allowed determination of the interacting residues or segments and also to delineate the sequence of events which occur in the CAM-peptide interaction. Calorimetric studies have also been carried out to understand the energetics of this process [24]. Upon the addition of calcium, hydrophobic surfaces get exposed, the two domains come closer and wrap themselves around the peptide and this peptide, which is otherwise unstructured in solution, adopts an α-helical conformation [14]. The energetic tradeoffs associated with this process, however, would be difficult to rationalize, as changes in the ion binding parameters have also been reported on effector peptide binding [25,26]. The binding affinity for calcium has been reported to increase, a feature which is not altogether surprising, as the protein needs to maintain the rigidity of its domains in order to effectively bind to the target segment.

The binding reaction seems to be driven by a cumulative effect of van der Waals/hydrophobic interactions in addition to the flexibility of the central linker. Evidence for the former stems from the fact that the smaller peptide, mastoparan exhibits exothermic heat of binding whereas the longer melittin shows an endothermic enthalpy. This can be rationalized in terms of the cost of dehydration of side chains that has to be paid before the binding event can occur. Thus for melittin, with the larger number of side chains, the binding event would be essentially endothermic. Despite the disparate nature of enthalpy changes associated with the binding of melittin and mastoparan, non-polar forces appear to be responsible for the interaction of the peptides with CAM as well as CaBP. That this is indeed the case is borne out by the large negative values of changes in heat capacities accompanying these reactions. If the correlation between ΔC_p and the water accessible surface area is taken [27], then approximately 2774.5 Å^2 would be buried when melittin binds to CaBP. Approximately, 1530.5 Å^2 and 1138.2 Å^2 surface area is sequestered when melittin and mastoparan, respectively, bind to CAM. The extent to which hydrophobic forces govern the recognition event was examined by performing ANS titrations on the protein-peptide complexes. The results demonstrate that CAM-melittin leaves less hydrophobic surface exposed after the binding as compared to CAM-mastoparan. The CaBP-melittin complex, on the other hand, seems to have most of its apolar surface exposed to the solvent even after the binding event has occurred and hence there is a little reduction in its ANS binding capacity. This could be related to the nature of the central linker in the two proteins. The flexible linking segment in CaBP might influence the positioning of the amino and carboxy terminal domains and hence hinder their tethering during the binding event. This interpretation is further supported by the fact that the shorter peptide, mastoparan, is unable to bind to CaBP, perhaps due to its inability to cover the entire stretch of the binding regions as the domains 'wobble' and adopt varied conformations with respect to each other.

The known crystal structure of CAM with smMLCK was used as a basis to propose possible models for the CaBP-peptide complex. Fig. 4A depicts one of the models wherein the N terminal domain in the two protein complexes are assumed to be in similar orientations and the extra residues in the linker are accommodated in the carboxy domain. Fig. 4B provides the alternate picture, namely, the C terminal domain contacts are by and large preserved (vis-a-vis CAM) and the amino terminal domain gets reoriented. This model seems less likely as the interactions are reduced to a greater extent. A third possibility is the one in which the increase in the length of the central linker gets accommodated within the loop region, with the domains maintaining relative orientations similar to that of CAM. This model, however, is not entirely appropriate as it fails to account for the observation that greater lengths of the peptide are needed for binding to CaBP.

In conclusion, we believe that the ability exhibited by CAM to bind to both the model peptides arises from the larger exposure of hydrophobic surfaces upon binding to calcium as well as a more rigid linker compared to CaBP.

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