Eur. J. Biochem. 243, 42–48 (1997)

Modified *helix*-loop-helix motifs of calmodulin

The influence of the exchange of helical regions on calcium-binding affinity

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(Received 20 June/30 October 1996) – EJB 96 0912/3

The four calcium-binding sites, called the helix-loop-helix, or the EF-hand motifs, of calmodulin differ in their ion-binding affinities; this has been thought to arise due to the variations in the sequences of the loop regions where the ion binds. We focus attention here on the role of the flanking helical regions on the calcium-binding affinities. Peptides were synthesized in a manner that simulates the E and F helical flanks of site 4 (the strongest calcium-binding site of the calmodulin) to sandwich the loop sequences of sites 1, 2, 3 and 4 so as to produce peptides named 414, 424, 434 and 444, as well as using the helical flanks of site 1 (the weakest site) to produce peptides 111, 121, 131 and 141. Calcium binding was monitored using the calcium-mimic dye Stains-all (4,4,4′,5′-dibenzo-3,3′-diethyl-9-methyl-thiacarbocyanine bromide). Binding abilities were seen to increase several-fold when the E and F helices of site 1 were replaced by those of site 4 (i.e., 111–414). In contrast, the intensity of circular dichroism induced in the absorption bands of the bound achiral dye decreased significantly when the helical flanks of site 4 were replaced with those of site 1 (i.e., 444–141). The helical flanks of site 4 impart greater binding ability to a given loop region, while the helical flanks of site 1 tend to weaken it.

Keywords: calmodulin; helix-loop-helix motif; 4,4,4′,5′-dibenzo-3,3′-diethyl-9-methyl-thiacarbocyanine bromide; synthetic peptide; molecular modeling.

Calmodulin, a highly conserved regulatory protein, consists of four homologous domains, each containing a single calcium-binding site. Binding sites 1 and 3 share sequence and structural similarities, as do sites 2 and 4. Site 4 has the highest affinity for calcium and site 1 the lowest (Klee and Vanaman, 1982; Teleman et al., 1986). Each calcium-binding site has the characteristic EF-hand or helix-loop-helix motif (Kretsinger and Nockolds, 1973; Kretsinger, 1980), and the calcium ion binds to the central loop region containing a 12-residue sequence rich in anionic and polar amino acid residues. This ligating loop is flanked on the N-terminal side by an amphipathic α-helical segment, called the E helix, and on the C-terminal side by the F helix. While the lengths of the E and F helices vary somewhat, their conformations and amphipathic characters are maintained in the four sites (Szebenyi and Moffat, 1986; Strynadka and James, 1989).

More than 200 proteins belonging to the superfamily of EF-hand calcium-binding proteins are known (Heizmann, 1991). The calcium-binding abilities of these proteins vary over a range, just as seen in the four sites of calmodulin. With a view to understanding the factors governing calcium affinity, several studies have concentrated on the sequence of the loop regions. Peptides corresponding to the individual calcium-binding sites of calmodulin and related proteins have been synthesized, along with their variants, and metal-binding evaluated using a variety of methods (Reid et al., 1981; Gariepy et al., 1983; Marsden et al., 1988, 1989). Since the binding of calcium occurs in the loop region, these studies have particularly concentrated on varying the sequence of this 12-residue loop submotif region and monitoring the resultant alterations in affinity (Marsden et al., 1990; Reid, 1990; Shaw et al., 1991; Procyshyn and Reid, 1994).

These studies do not, however, answer the question of what the contribution of the other submotif, namely the flanking helices E and F might be towards calcium affinity. The length and the hydrophobic character of these helical submotifs appear to influence the calcium affinity (Reid, 1987; Sekharudu and Sundaralingam, 1988; Monera et al., 1992). A specific relationship between the α-helix stability and ion-binding affinity was suggested in troponin C by substitution of the N-cap residue of the G-helix (residue 130) by various amino acids (Trigo-Gonzalez et al., 1993). The amphiphilic nature of the helical segments would be expected to provide a relatively non-polar shield that would augment the binding efficiency of the loop on the one hand, and modulate binding-site geometry through steric interactions on the other (Szebenyi and Moffat, 1986).

In calmodulin, site 1 is thought to bind Ca^{2+} weaker than site 4, because of the two cationic residues in its loop region which would inhibit the approach of the metal ion; the sequence of the loop region of site 1 is DKDGDGTITTKE while that of site 4 is DIDGDGQVNYEE. In other words, what are the factors responsible for the large variation in their calcium affinities? But if we consider the above reports the question arises as to what the actual role of the flanking helices may be; for example, would replacing the helical region of site 1 by those of site 4

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Abbreviations. CVFF, consistent-valence force field; MD, molecular dynamics; Stains-all, 4,4,4′,5′-dibenzo-3,3′-diethyl-9-methyl-thiacarbocyanine bromide; PDB, Protein Data Bank.

(keeping the loop region intact) enhance the binding ability of site 1?

In an effort to consider this question, we have synthesized two sets of peptides corresponding to the regions of the calcium-binding sites of calmodulin. In the first set, the E and F helical regions of site 4 were used as the invariant flanking regions and the loop region varied. In the second set, the E and F helical regions of site 1 were used as flanks that sandwich the binding loops of sites 1, 2, 3 and 4. These generated eight peptides that we label as 414, 424, 434, 444 and 111, 121, 131 and 141. The actual sequences that were synthesized are shown in Fig. 1. Sites 1 and 4 were chosen for this study because, as mentioned earlier, site 1 of calmodulin displays the weakest affinity for calcium and site 4 the strongest.

When studying peptides of this length, direct assay methods of calcium binding, such as equilibrium dialysis, are rendered difficult because of the small size of the ligating substrate and the lower affinity. Spectroscopic methods involving calcium-ion mimics such as lanthanide binding, or dye binding, are therefore used. The use of the cationic carbocyanine dye Stains-all (4,4,4′,5′-dibenzo-3,3′-diethyl-9-methyl-thiacarbocyanine bromide) to monitor calcium-ion binding to calcium-binding peptides and proteins has been established (Caday et al., 1986; Sharma et al., 1989, 1993). This dye binds at the calcium-binding site, from where it can be displaced upon the addition of excess calcium (Caday and Steiner, 1985). This spectral assay is easy, accurate and conformation sensitive (Sharma and Balasubramanian, 1991). We have used the Stains-all binding assay to monitor the influence of the helical submotifs of site 1 and of site 4 on the four different calcium-binding loops of calmodulin. The results obtained from studies on the relative affinity of the peptides towards calcium have been compared with simulated models of these peptides generated using a molecular dynamics approach.

EXPERIMENTAIL PROCEDURES

Stains-all and Mops were purchased from Sigma. All other chemicals and reagents used were commonly available and were of analytical grade, or better.

Peptide synthesis. All the peptides were synthesized by the Merrifield solid-phase method (Merrifield, 1969) with some modifications (Fairwell et al., 1987), using a Millipore 9600 automated peptide synthesizer and a modified *t*-butoxylcarbonyl (Boc) protocol. Amino acids were protected at the side chains using benzyl (Thr, Ser), 2-chlorobenzyloxycarbonyl (Lys), *p*-toluenesulfonyl (Arg), cyclohexyl (Glu, Asp), and 2-bromobenzyloxycarbonyl (Tyr) moieties. Double coupling was employed at every step and 1-hydroxybenzotriazole was used as the coupling catalyst. After hydrogen fluoride cleavage, the crude peptides were purified by reverse-phase HPLC using a Perkin Elmer HPLC unit equipped with a diode-array detector and a Vydac C18 column. The purity and homogeneity of each peptide was established by automated Edman sequencing, as well as by electrospray-ionization MS, using a Finnigan TSQ 700 mass spectrometer.

Stains-all binding assays. All peptides and dye solutions were made in 2 mM Mops, pH 7.2, containing 30% ethylene glycol as described earlier (Caday and Steiner, 1985). The CD spectra were recorded on a Jasco J-20 spectropolarimeter at room temperature. The peptides were added to the dye solution, incubated for 5 min and CD spectra recorded using cells of 1 cm or 2 cm pathlength. Molar ellipticities were expressed in terms of dye concentration. The dye concentration was kept constant (22 μM) in every case so as to aid direct comparison. The dye/peptide ratio was kept around 1:4 (22 μM:90 μM) in the case of peptides 111, 121, 131 and 141, which displayed the weaker binding. The other peptides were used at lower concentrations, ranging from 3 μM (peptide 444) to 30 μM (peptide 424).

Calcium-binding experiments. Far-ultraviolet CD spectra of the peptides were recorded in 50 mM Tris, pH 7.4 on a Jobin Yvon (Mark V) spectropolarimeter using a 0.05-cm-pathlength cell. Changes in ellipticity at 222 nm were measured following the addition of calcium to the peptides. Mean residue ellipticity and fractional changes in ellipticity were calculated as described earlier (Reid, 1987).

To determine the extent of dimer formation HPLC runs of the peptides were performed on a Waters I-60 gel filtration column (flow rate, 1 ml/min). All samples were prepared in 2 mM Mops, pH 7.2, containing 30% ethylene glycol and equilibrated in the required amounts of calcium for 1 h prior to the run.

Molecular modeling. Molecular simulations were performed on peptides constructed using coordinates for a calmodulin model available in the Brookhaven Protein Data Bank (PDB; Babu et al., 1985, 1988). The backbone coordinates for peptide 111 were used as the starting conformations for peptides 141, 131 and 121; those for peptide 444 served as starting conformations for peptides 414, 424 and 434. The software used for all simulations was from Biosym Technologies, San Diego, and was executed on a Silicon Graphics Iris Crimson computer. Optimization and dynamics routines were performed using the Discover program, and graphical displays generated with the InsightII molecular-modeling system. The structures were first subjected to an optimization procedure involving limited minimization with a variety of protocols in succession, followed by molecular dynamics (MD) *in vacuo* at 300 K for 2 cycles of 10 ps each. Low-energy structures from near the end of each MD operation were chosen, averaged and used for succeeding steps. After the second MD operation, the structures were optimized again. In the case of calcium-bound peptides, the calcium atom was constrained at its PDB coordinates, as were six oxygens (five side-chain O atoms and one backbone O atom) (McPhalen et al., 1991). Potentials were fixed using consistent-valence force field (CVFF).

For the peptide dimer simulations, two copies of the starting structures were arranged, head to tail, with the loops parallel to each other. The orientation was similar to that adopted by adjacent helix-loop-helix motifs in the native calmodulin molecule, except that the peptides here were placed such that the closest distance between two atoms on the adjacent peptides marginally exceeded 0.6 nm. One of the two peptides was tethered in space by positional fixing of the calcium atom and the oxygens that coordinate with it. Optimization and MD operations were performed on this assembly as described above.

RESULTS AND DISCUSSION

Design, synthesis and purification of peptides. We have synthesized and studied EF-hand peptides corresponding to the four calcium-binding loops of calmodulin, linked in all permutations to the flanking helices of the weak calcium-binding site 1 and the strong site 4. The E helices adjoining the different loops in the actual protein molecule vary in length. There are conflicting views on the length of the E helices (Vanaman, 1983; Babu et al., 1988). X-ray studies reveal that the E helices of sites 2 and 4 are 11 residues long, while that of site 3 is 17 residues long and that of site 1 is 15 residues long. The length of the F helix in site 4 is only 8 residues from the end of the binding loop, and the EF-hand motif is 29 residues long. For proper comparison of the role of helices, we chose to keep the helix length at eight

Peptides	amino acid sequence
p111	^{12}FKEAFSLF**DKDGDGTITTKE**LGTVMRSL^{39}
p121	FKEAFSLF**DADGNGTIDFPE**LGTVMRSL
p131	FKEAFSLF**DKDGNGYISAAE**LGTVMRSL
p141	FKEAFSLF**DIDGDGQVNYEE**LGTVMRSL
p414	VDEMIREA**DKDGDGTITTKE**FVQMMTAK
p424	VDEMIREA**DADGNGTIDFPE**FVQMMTAK
p434	VDEMIREA**DKDGNGYISAAE**FVQMMTAK
p444	^{121}VDEMIREA**DIDGDGQVNYEE**FVQMMTAK^{148}

Fig. 1. The calmodulin peptides and their variants with helices from sites 1 and 4 synthesized for this study.

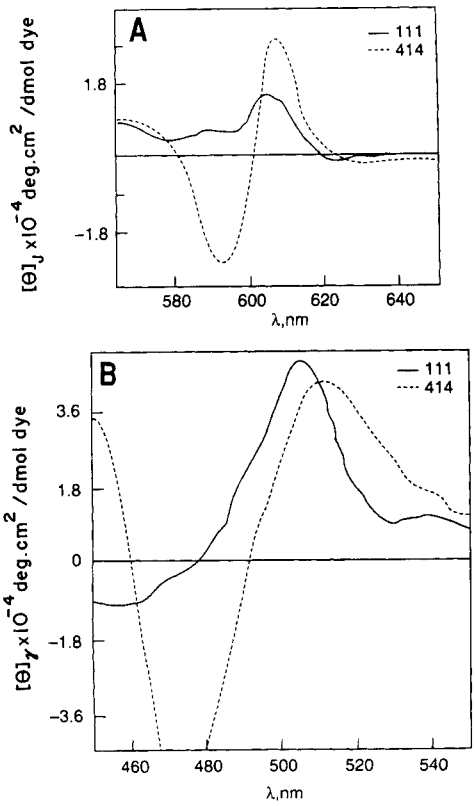

Fig. 2. CD spectra of the Stains-all complex (A) in the J band and in (B) γ band region with EF-hand motif belonging to calmodulin peptide 111 (——) and the modified peptide 414 (– – –) in 2 mM Mops, pH 7.2, containing 30% ethylene glycol. Dye concentration used was 22 μM. The ratio of the concentration of peptides 414/111 was about 1:30.

residues on either side of the binding loop. As a result, all the peptides synthesized for this study are 28 residues long. Six chimeric peptides with the exchange of helices of these sites were also synthesized as shown in Fig. 1.

Calcium binding as monitored by Stains-all assay. We have compared calcium affinities of the peptides by monitoring the induced CD spectral intensities (ellipticities) of the dye Stains-all, which is inherently achiral or optically inactive. CD is induced in such cases only upon binding to the chiral surface of the substrate peptide; also, Ca^{2+} addition displaces the dye and

Table 1. Normalized relative ellipticities induced in Stains-all by equal concentrations of various peptides. Stains-all concentration used was 22 μM. The values are normalized for identical concentrations (90 μM, dye/peptide ratio of 1:4), in order to afford direct comparison.

Peptides	$10^4 \times [\theta]$	
	J band	γ band
	deg · cm^2 · dmol dye^{-1}	
111	1.5	4.0
414	85.0	110.0
121	1.0	4.0
424	10.0	–
131	–	−1.0
434	6.4	−1.6
141	2.6	5.5
444	65.0	36.0

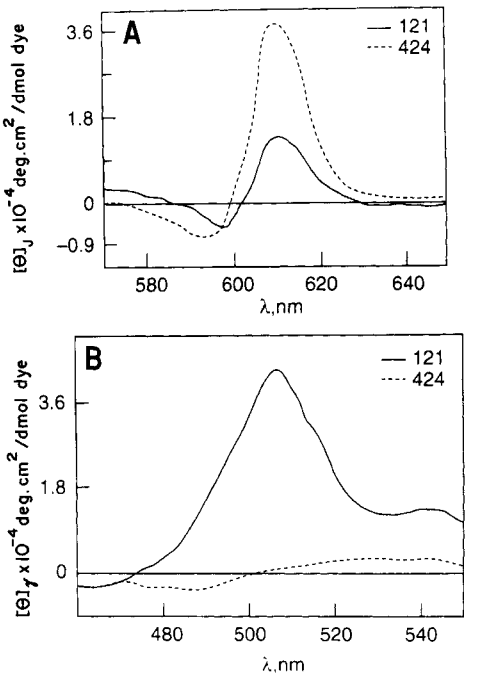

Fig. 3. CD spectra of the complex of EF-hand peptides 121 (——) and 424 (– – –) with Stains-all in 2 mM Mops, pH 7.2, containing 30% ethylene glycol (A) in the J band, and in (B) the γ band region. Dye concentration used was 22 μM. The concentration ratio of peptides 424/121 was 1:3.

abolishes the induced CD bands. This method is particularly suited for comparing the calcium affinities of closely related peptides (Sharma et al., 1993). Exchange of the helices flanking site 4 with those of site 1 and vice versa is seen to lead to a significant change in the affinity of peptides to Stains-all as monitored by the magnitude of induced CD in the J band region (Figs 2–5). Table 1 summarizes the relative abilities of the various peptides in interacting with Stains-all and inducing CD bands in the J and/or γ band regions of the dye, at comparable concentrations of the peptides and at constant dye/peptide ratios. The salient results can be summarized as follows.

Replacing the E and F helices flanking site 1 with those of site 4 (i.e., modifying peptide 111 into 414) leads to a 60-fold increase in affinity to Stains-all. Peptide 111 is not very effective in inducing the J band whereas 414 induces this band efficiently (Fig. 2A). Likewise 414 effects a 30-fold increase in the elliptic-

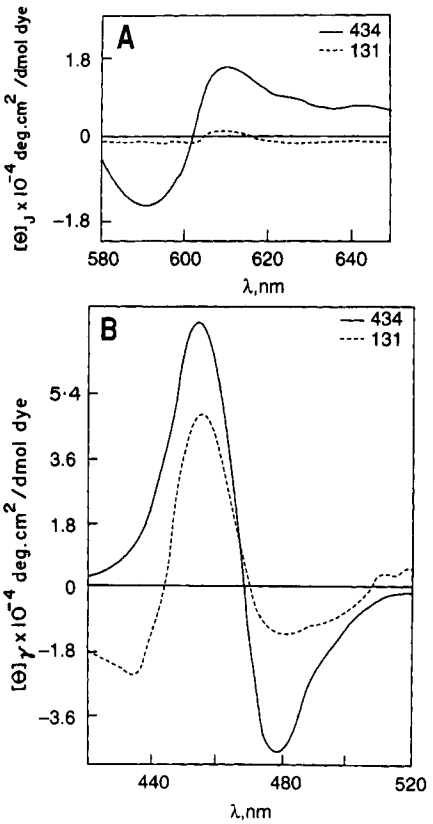

Fig. 4. CD spectra of the complex of EF-hand peptides 434 (——) and 131 (– – –) with Stains-all in 2 mM Mops, pH 7.2, containing 30 % ethylene glycol in the (A) J and (B) γ band regions. Dye concentration used was 22 μM. The concentration ratio of peptides 434/131 was 1:4.

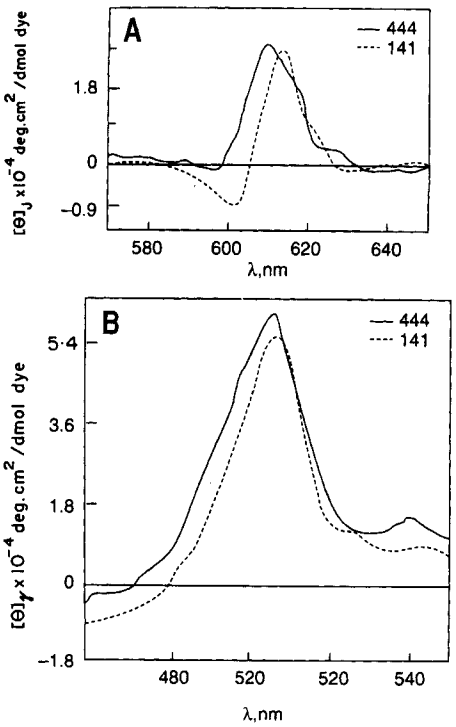

Fig. 5. CD spectra of the complex of EF-hand peptides 444 (——) and 141 (– – –) with Stains-all in 2 mM Mops, pH 7.2, containing 30 % ethylene glycol. Dye concentration used was 22 μM. The concentration ratio of peptides 444/141 was (A) 1:25 and (B) 1:6.

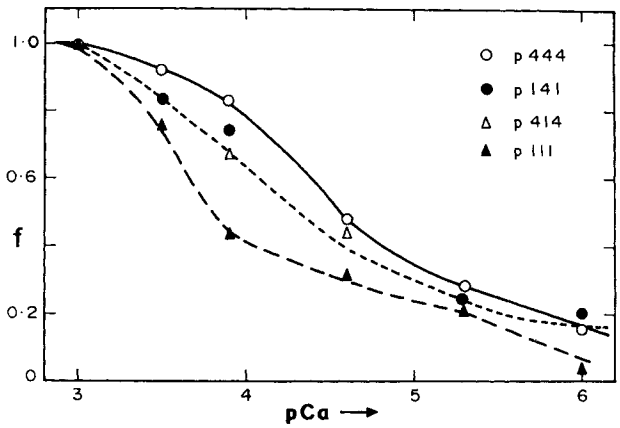

Fig. 6. CD monitored calcium titration of Calmodulin peptides, p111, p414, p141, and p444. f is the ratio of the calcium-induced change in ellipticity at 222 nm to the maximum change in ellipticity that can be induced by calcium. pCa is the negative logarithm of Ca^{2+} concentration. The far-ultraviolet CD spectra were run in 50 mM Tris, pH 7.4, with the concentration of peptides kept at 16 μM in each case.

ity of the γ band of the dye, in comparison to peptide 111 (Fig. 2B; Table 1).

In the case of loop 2 (peptides 121 and 424), peptide 424 enhances the J band of the dye by a factor of ten. Here again, the flanking helices of site 4 can induce a stronger band than those of site 1. However, the peptide 424 failed to induce a γ band, (Fig. 3) suggesting a possible conformational readjustment.

Comparison of peptides 131 and 434 shows that peptide 131 could not induce the J band at the concentrations used, while it is distinctly induced by peptide 434 (Fig. 4A). The γ band is induced, but in contrast to the other sites, the CD bands here are of opposite signs (Fig. 4B). Peptide 333 (site 3) itself only weakly binds the dye, and modifying it to 131 seems to weaken it further. Modification to 434 improves the binding notably (Fig. 4B). However, we found in other experiments that dye binding is induced in an extended version of peptide 333, where the number of residues is increased to 32, two extra amino acids each being added to the flanking helices (data not shown). In calmodulin, site 3 has a high affinity for calcium, which is in contrast to our data on the isolated site 3 fragment. It has been suggested that site 3 in calmodulin experiences cooperative ligand-binding effects from the other sites (Linse et al., 1991).

The inducibility of dye bands by peptide 141 is greatly reduced in relation to peptide 444. Peptide 444 induces a 25-fold greater ellipticity of the J band of Stains-all than peptide 141 (Fig. 5A, Table 1). It is important to note that the dye aggregates at very high concentrations of peptide, making it difficult to reach the limit, or plateau. For inducing J bands of similar magnitudes, 1 μM peptide 444 or about 25 μM peptide 141 are required. In the γ band region too, the ability of peptide 141 to induce ellipticity decreases sixfold (Fig. 5B, Table 1).

Calcium-binding as monitored by ellipticity at 222 nm. In calmodulin and other related calcium-binding proteins, addition of calcium ions induces conformational changes by altering the helical content, which can be seen in the far-ultraviolet CD (Klee et al., 1980). Change in the ellipticity at 222 nm with calcium addition can be used to compare the affinity of peptides. Four peptides (p111, p414, p141 and p444) were titrated with calcium until no further change in ellipticity at 222 nm was observed. The data are represented as a fraction f (change in ellipticity

measured at 222 nm at various calcium concentrations, divided by the maximum change induced by calcium) plotted against pCa, the negative logarithm of calcium concentration (Reid, 1987) in Fig. 6. A 2–3-fold higher concentration of calcium is required to induce half the maximum change in ellipticity in peptide 111 compared with peptide 444. This is indicative of the difference in the affinities of the peptides for calcium. The affinity for calcium decreases in the order 444 > 141≈ 414 > 111. These results substantiate the data on the binding of Stains-all to these peptides. The calcium affinities of peptides 141 and 414 are so close, that only by Stains-all binding could it be shown that peptide 141 may have a higher affinity than peptide 414.

Helicity of peptides. The mean residue ellipticity of a peptide at 222 nm is an indicator of its helical content. The mean residue ellipticities of the apo-peptides at 222 nm differ considerably, being significantly higher in the case of peptides with helices from site 4, and lower in peptides where helices from site 1 are attached, even though the length of the helices are kept constant in all peptides. There is a difference in the mean residue ellipticities of peptides 444 and 414, as well as peptides 141 and 111, though these peptide pairs share the same helices. This difference in ellipticity of peptides with common helices ought to be due to the influence of the loop regions. This is also evident in molecular-modeling studies where the E helix in peptides with the loop of site I opens up partially (Fig. 7A). This suggests that a helical motif adjoining a loop is not a sufficient criterion for determining affinity, but the sequence of the helices (site 1 type, or site 4 type) plays an influencing role in the stability and/or geometry of an EF-hand as well as its affinity for calcium, in addition to those of the loop region.

Molecular simulations. A model-building exercise was undertaken to compare the sequences described here. Using the crystal coordinates (Babu et al., 1988), peptide fragment corresponding to 111 and 444 were 'cut' out. The sequences for 121, 131 and 141 were generated on the 111 backbone template; the fragment corresponding to 444 served as the template for 414, 424 and 434. This method of inducing 'mutations' in topologically equivalent positions was adopted because of the close similarity in the structure of the motif between the different sites, and the identical number of residues: in the crystal structure, the rms deviation between the positions of backbone atoms corresponding to peptides 111 and 444 is 0.1 nm, that between 111 and 333 is 0.033 nm. Simple energy minimizations of the simulated peptides resulted in conformations that matched well with the helix-loop-helix regions in the protein crystals; the newly introduced amino acids did not lead to deformations in backbone structure due to steric reasons.

Following MD and optimization procedures, we found that the rmsd of bond angles amongst atoms of the backbone, in all the peptides studied, varied from their original states within a limited range (2.4°–2.9°), while the Ramachandran plots describing the backbone dihedral angles showed that almost all amino acid residues maintained regular conformations. Empirical energy calculations showed that peptide 444 had the lowest total energy in relation to the other peptides and peptide 111 had the highest, with the order 444 < 141 < 414 < 111 (data not shown).

Verification of structures with regard to hydrogen-bond positions indicated that the F-helix portion in all the peptides had a saturation very similar to that seen in the template peptides, indicating a minimal deviation from the original motif. The ribbon diagrams of these peptides (Fig. 7A) reveal the preservation of the F helix in all the peptides. However, the E helix shows a tendency to open up, this tendency being more pronounced in

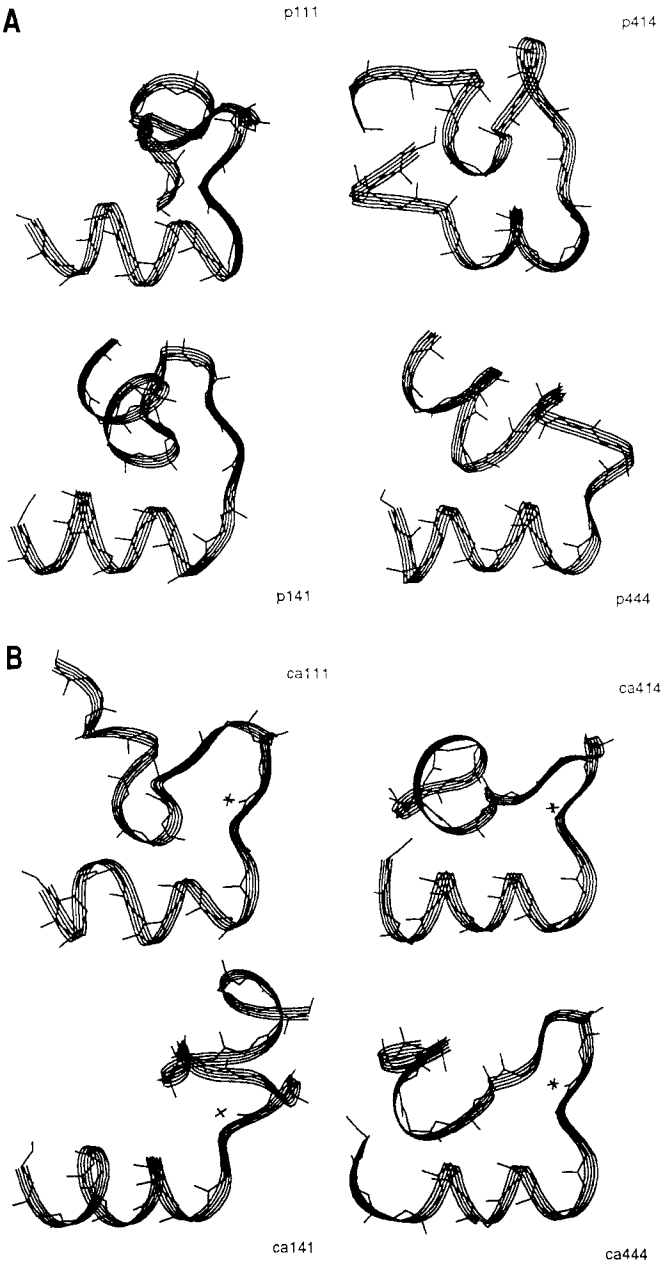

Fig. 7. Ribbon diagrams of peptides simulated in the (A) absence and (B) presence of calcium. All figures have been aligned with the F helix base of the frame.

111. We also performed energy minimization and MD protocols on the calcium-bound forms of these peptides: the presence of the calcium ion leads to a conformational readjustment (Fig. 7B). These results are in accord with what was observed in the CD experiments.

A point of special interest is evident when one compares the overlays of these structures. While the E and F (amphiphilic) helices of 444 close in on the loop region, with their hydrophobic sides facing inward, the flanking E helix of 111 is relatively flung out of the loop region. The increase in the binding ability of 444 vis-a-vis 111 may be rationalized based on the relative dispositions of the flanking helical regions in them. Some degree of helicity is retained in the E helix of both peptides, 414 and 141, but the helix in 141 is tilted away, as is apparent in the lower axis, Z-aligned view. These results suggest that the spatial dispositions and the ability to protect the loop region through the hydrophobic faces of the E and F helices augment the bind-

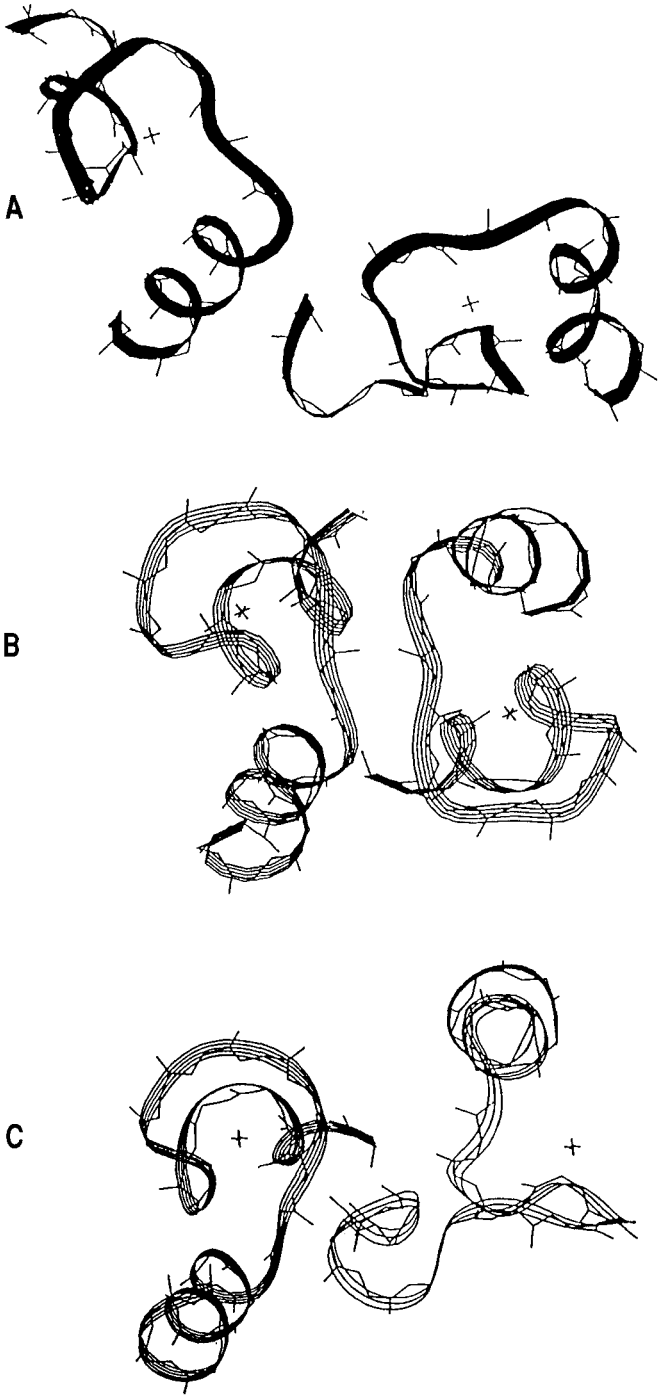

Fig. 8. Ribbon diagrams of dimer simulations of calcium-containing calmodulin peptides (A) p111; (B) p444; (C) p444, and p414. In all cases, the peptide on the left was tethered, as described in Experimental Procedures.

ing ability of individual binding sites of the calmodulin class of calcium-binding proteins. The calcium-free form of 444 possesses the highest degree of helicity, and peptide 111 the least. Together with the calcium-affinity experiments, these results suggest that the helical content of these peptides has a strong influence on their affinity towards calcium. Curiously, a comparison of model peptides 144 and 441, both built on the 444 template, indicated that 441 would be the stabler entity, both in measured energy terms and in terms of helical conformation (data not shown).

Calcium binding and dimer formation. Finally, we take a brief diversion to the issue of dimerization of this class of calcium-binding peptides of calmodulin. Earlier reports have shown that peptides corresponding to the helix-loop-helix motif of the related calcium-binding protein, troponin C, tend to form symmetric two-site calcium-binding domains, aligned head-to-tail, in the presence of calcium (Kay et al., 1991; Monera et al., 1992). In the HPLC runs of our peptides in increasing concentrations of calcium (0, 25 and 50 mM), peptide 111 eluted as a single peak. However, in the case of peptide 444, with calcium, a leading shoulder was noticeable in the gel-filtration eluate, indicative of the presence of some amounts of a higher-molecular-mass aggregate. We surmise that this shoulder corresponds to a dimer of the peptide. Earlier reports, with a 34-amino-acid synthetic peptide corresponding to calcium-binding site 3 of troponin C (Shaw et al., 1990), and a 39-amino-acid proteolytic fragment of site 4 of troponin C (Kay et al., 1991) have indicated that these peptides form dimers in the presence of calcium. The dimer-forming tendency of the 28-amino-acid site 4 peptide of calmodulin is not strong; under conditions used in this study, only 50 mM Ca^{2+} could generate a dimer. Peptide 141 did not show any dimer-forming tendencies, and the magnitude of the leading shoulder for peptide 414 was smaller than that obtained for 444. The dimer formation is favored at the higher peptide concentrations and with high concentrations of calcium ion.

We have also simulated dimer formation by placing the starting structures of the peptides in orientations similar to those seen in the native proteins. Under these conditions, the two subunits of peptide 111 tended to drift apart and lose symmetry (Fig. 8A). Peptide 444 underwent a compaction, leading to the formation of a symmetric dimeric entity, held together by hydrophobic interactions (Fig. 8B). The geometry of this dimer was very similar to that seen for adjacent calcium-binding sites in single domains of calmodulin and other similar calcium-binding proteins. However, heterodimeric simulations, using peptides 414 and 444, did not lead to the formation of a compact unit (Fig. 8C). It would thus appear that the contribution of the helix towards dimer formation is not total. The weak calcium affinity of site I of calmodulin may be linked to the weak dimerizing ability of this site.

Calcium-binding proteins are known to contain helix-loop-helix, or EF-hand motifs of the type 111 or 444. A search in the SwissProt database failed to yield sequences identical or even nearly identical to the hybrid structures described in this study (Fig. 1). The closest match obtained was between peptide 131 and the calcium-binding site I in calmodulins from several species of plant, which differ from peptide 131 at five positions, all in the loop region; four replacements being with similar amino acids and one being dissimilar, e.g., the sequence F(12)KEAFSLFDKDGDGCITTKELGTVMRSL in the calmodulin from *Medicago sativa* (Barnett and Long, 1990).

This work was done under the National Institutes of Health–Centre for Cellular and Molecular Biology collaborative programme. D. Balasubramanian thanks the Jawaharial Nehru Centre for Advanced Scientific Research, Bangalore, of which he is an honorary professor.

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Supplementary material. **Modified helix-loop-helix motifs of calmodulin. The influence of the helical regions on calcium-binding affinity.** Fig. S1. Stereo representations of overlaid ribbons of peptides p111 and p444; p111 and p414; and p414 and p444. Fig. S2. HPLC profiles of calmodulin peptides run on an I-60 gel-filtration column. Table S1. Molar ellipticity values of the synthetic calmodulin peptides 111, 414, 141 and 444. This information is available, on request, from the Editorial Office. Six pages are available.