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A Synthetic 33-Residue Analogue of Bovine Brain Calmodulin Calcium Binding Site III: Synthesis, Purification, and Calcium Binding†

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ABSTRACT: The sequential solid-phase synthesis of a peptide analogue of bovine brain calmodulin calcium binding site III covering residues 81–113 of the natural sequence is described. Methionine-109 is replaced by a leucine residue to avoid complications in the synthesis and purification. In an attempt to relate the structure of the calcium binding sites in the naturally occurring calcium binding protein to the calcium affinity of these sites, the synthetic analogue is examined for calcium binding by circular dichroism spectroscopy. The calcium binding characteristics are compared to those of a synthetic analogue of the homologous calcium binding site III in rabbit skeletal troponin C. The K_d of the calmodulin site III fragment for Ca^{2+} is determined as 878 μM whereas the K_d of the troponin C fragment is 30 times smaller at 28 μM. Structural changes induced in the peptides by Ca^{2+} and trifluoroethanol are similar. This study supports our contention that the single synthetic calcium binding site is a reasonable model for the study of the structure–activity relationships of the calcium binding sites in calcium-regulated proteins such as calmodulin and troponin C.

The function of calmodulin (CaM)[1] at the molecular level has proven to be an active area of inquiry [for a recent review, see Cox et al. (1984)]. A description of the mechanism by which calmodulin can regulate several different enzyme systems, some of which are mutually antagonistic, in response to changes in intracellular calcium levels, is a major barrier to be overcome. Current thought on the subject suggests that calcium binds to the four sites on CaM with differing affinities resulting in a stepwise change in structure dependent on calcium concentration. Different enzymes recognize different conformations of CaM and are thereby differentially regulated depending on the calcium concentration of the medium (Klee et al., 1986). Since difficulties can arise in the interpretation of experimental results from studies on the natural protein, which binds 4 mol of calcium/mol of protein, several authors have attempted to simplify the experimental conditions by reducing the number of calcium binding sites through the use of fragments of the natural protein obtained by controlled enzymic cleavage (Newton et al., 1984; Ni & Klee, 1985; Thulin et al., 1984; Malencik & Anderson, 1984; Dalgarno et al., 1984; Krebs et al., 1984; Guerini et al., 1984; Ikura et al., 1984; Minowa & Yagi, 1984; Aulabaugh et al., 1984; Wall et al., 1981; Kuznicki et al., 1981; Head et al., 1982; Brzeska et al., 1983; Vogel et al., 1983). Although several sound hypothetical arguments can be put forward against extrapolating from fragment studies on a protein to the biological function of the intact protein, many areas of research such as synthetic antigens (Atassi, 1984) and hormone analogues (Gysin & Schwyzer, 1984) have shown that such studies contribute substantially to an understanding of the biological

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[1] Abbreviations: CaM, calmodulin; TnC, troponin C; DIEA, diisopropylethylamine; DMF, dimethylformamide; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenztriazole; Tris, tris(hydroxymethyl)-aminomethane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Boc, *tert*-butyloxycarbonyl; TFA, trifluoroacetic acid; TFE, trifluoroethanol; IE-HPLC, ion-exchange high-pressure liquid chromatography; RP-HPLC, reversed-phase high-pressure liquid chromatography; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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function of the natural protein.

Fragments prepared by chemical synthesis are an alternative to fragments prepared by enzymic cleavage of the natural protein. Studies on synthetic fragment analogues of calcium binding proteins such as calmodulin, troponin C, and parvalbumin have not been as abundant in the literature as those of the fragments obtained from the proteins. Short synthetic fragments corresponding to the loop region of calcium binding helix–loop–helix units in calmodulin have been prepared and show little or no affinity for calcium (Buchta et al., 1986; Pavone et al., 1984; Borin et al., 1985; Marchiori et al., 1983), but the cation affinity can be increased by use of lanthanide cations in place of calcium (Buchta et al., 1986) or by use of a buffer containing trifluoroethanol.

Earlier studies on larger synthetic fragments of the high-affinity calcium binding site III of rabbit skeletal troponin C indicated that the calcium affinity in aqueous buffer could be increased by extension of the α-helical segments in the synthetic peptides (Reid et al., 1980, 1981). A major problem that severely curtailed these TnC fragment studies was the inability to extend the solid-phase sequential synthesis beyond the 20-residue stage without significant loss of peptide (Reid et al., 1981). In order to synthesize the 34-residue peptides, it was necessary to prepare fully protected tri- and tetrapeptide fragments in solution and couple these fragments to a 20-residue fragment prepared on the resin by sequential synthesis. This introduced further complications due to the solution synthesis of small fragments. The problem has been overcome through the development of a protocol that results in total sequential synthesis of 33- and 34-residue calcium binding peptides (Reid, 1987).

This paper describes the complete sequential solid-phase synthesis and high-pressure liquid chromatographic purification of an analogue of bovine brain calmodulin calcium binding site III covering residues 81–113 of the natural sequence. Calcium binding by the peptide is examined with CD spectroscopy and compared to that of the synthetic analogue of the homologous calcium binding site III of rabbit skeletal troponin C.

Experimental Procedures

Circular Dichroism Spectroscopy. The CD spectroscopic measurements were made on a JASCO J500A CD spectrophotometer.

Free calcium concentrations were determined with a modified Perrin and Sayce computer program (Perrin & Sayce, 1967) with $\log_{10}$ of the association constants for the complexing species of EGTA (Sillen & Martell, 1964) set at the following values: H^+ to $EGTA^{4-}$, 9.46; H^+ to $HEGTA^{3-}$, 8.85; H^+ to H_2EGTA^{2-}, 2.68; H^+ to H_3EGTA^-, 2.00; Ca^{2+} to $EGTA^{4-}$, 11.00; Ca^{2+} to $HEGTA^{3-}$, 5.33. The free metal concentration corrected for peptide-bound calcium is obtained by reiteration with a starting value of 1:1 stoichiometry for the Ca–peptide complex. The procedure is repeated until the calculated association constant equals that inserted in the Perrin and Sayce program. The H^+ to peptide $\log_{10}$ association constant was set at 4.00 to correspond roughly to the pK_a of the acid side chains directly involved in calcium chelation. The association constant of peptide for calcium was calculated with a nonlinear regression computer program that fit the CD calcium titration data to

$$f = \frac{K_{Ca}[Ca^{2+}]}{1 + K_{Ca}[Ca^{2+}]}$$

where f is the fraction of peptide molecules in the calcium chelated state and is determined as the ratio of the change in ellipticity at 222 nm to the maximum change in ellipticity that can be ellicited by calcium at 222 nm. K_{Ca} is the apparent association constant of the peptide for calcium, and $[Ca^{2+}]$ is the concentration of free calcium calculated as described above. This calculation assumes that all activities are equal to concentrations and that the peptide has a single independent calcium binding site.

A previous study (Nagy et al., 1978) has shown the α-helix content from $[\theta]_{222}$ for a pure α-helix with an added refinement allowing for lengths of helical segments (Chen et al., 1974) can be used to arrive at an α-helix content for a fragment of CaM in the presence and absence of Ca^{2+} and that the difference between these two values is in good agreement with the value derived from the CD difference spectrum by the curve fitting procedure. Hence, this simplified procedure was adopted to estimate the helical content of the synthetic peptides. The fraction of α-helix (f_h) present was calculated as

$$f_h = \frac{[\theta]_{222}}{[\theta]_H(1 - k/\bar{n})}$$

where $[\theta]_H$ and k are calculated constants (Chen et al., 1974), which are 39 500° and 2.57, respectively, and $\bar{n}$ is the average helical length, taken as 9 in this case (Reid et al., 1981; Nagy et al., 1978).

The CD studies were carried out in a 100 mM MOPS, pH 7.2/150 mM KCl/1 mM EGTA buffer and a 1/1 (v/v) mixture of this buffer with trifluoroethanol. The calcium-free solutions were prepared from deionized water prior to use, and Nalgene laboratory equipment was used in place of glassware to avoid contamination of solutions with calcium leached from glass. Concentrations of the calcium solutions used in the titrations were determined by titration with EGTA as the primary standard, murexide being used as indicator. The peptides were quantitated from amino acid analysis of an aliquot of the solution.

Results and Discussion

A major feature of the amino acid sequence of the helix–loop–helix units in rabbit skeletal TnC is that the two high-affinity calcium binding sites have four acid residues in the coordinating positions and the acids are paired on X and Z coordinates of the octahedral arrangement of coordinating residues (Reid & Hodges, 1980). The two sites exhibiting low affinity also have four acid residues in coordinating positions, but only two of these residues are paired on the octahedral coordinates. Our working hypothesis is that pairing of the acid residues allows the negatively charged residues to approach the cation from opposite sides, hence maximizing approach to the cation while minimizing repulsion of the like charges. Therefore, the high-affinity sites have the repulsion reduced as much as possible, but the low-affinity sites still have the problem of repulsion of like charges (Reid & Hodges, 1980). While it is obvious that the high- and low-affinity sites on TnC can be separated on this basis (Reid & Hodges, 1980), the sequences in bovine brain calmodulin do not display a clear separation of affinity for calcium. The outstanding feature in this case is the lack of any acid pairs in site III (Figure 1). On the basis of this observation, calcium binding site III of bovine brain calmodulin should be low affinity for calcium.

The main result of this study is the large difference in the calcium dissociation constant of $AcL^{109}CaM(81–113)amide$ (878 μM) when compared to the sequentially homologous fragment of rabbit skeletal troponin C calcium binding site III, $AcA^{98}STnC(90–123)amide$ (28 μM) (Figure 2) (Reid, 1987). This result is consistent with the explanation that the

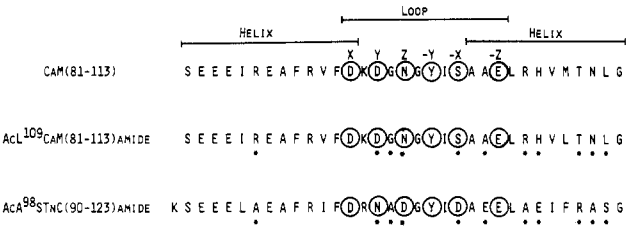

FIGURE 1: Amino acid sequence of CaM(81–113), AcL^{109}CaM(81–113)amide, and AcA^{98}STnC(90–123)amide. N- and C-terminal helical regions flanking the calcium binding loop region are indicated by horizontal bars. The amino acid side chains in the loop region interacting with calcium are circled, and the position in the octahedral arrangement of ligands is indicated. Asterisks indicate the positions of major differences in the amino acid sequence of the two peptides.

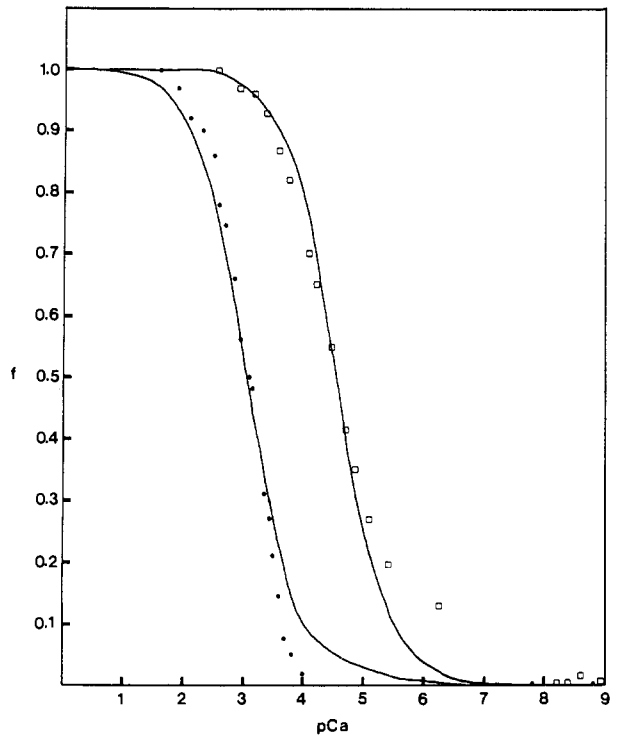

FIGURE 2: CD-monitored calcium titration of AcL^{109}CaM(81–113)amide (●) and AcA^{98}STnC(90–123)amide (□). f is the ratio of the calcium-induced change in ellipticity at 222 nm to the maximum change in ellipticity that can be elicited by calcium at 222 nm. The solid lines are the computer-generated fits described under Experimental Procedures.

TnC site III analogue contains five acidic side chains in the loop sequence, four of which are paired on the apical vertices of an octahedral arrangement of chelating ligands. The fifth acid residue is not considered to be a ligand for calcium. CaM site III contains three acidic residues, all of which chelate calcium and none of which are paired on apical vertices of the octahedral arrangement of chelating ligands (Figure 1). The higher K_d for calcium may be attributed to the impossibility of having two anionic residues approach the cation from opposite sides, maximizing interaction and minimizing repulsion. If two acid residues are to interact synergistically with Ca^{2+}, as suggested for the TnC peptide, they must do so at right angles to one another, and thus, a factor involving repulsion of the like charges may hinder interaction with the cation. It is also possible that the higher K_d for this peptide is due to the fact that there are only three anionic acidic residues in chelating positions compared to four in the TnC peptide. This possibility is currently under investigation.

The difference in C-terminal α-helix sequence may also contribute to a significant difference in dissociation constant between the two peptides. There are 11 positions in the amino acid sequences of these peptides that could be considered significant replacements (see asterisks in Figure 1). Of these 11 differences, 7 occur in the α-helical regions, and 6 of the 7 occur in the C-terminal α-helix. By use of the Chou and Fasman method of conformation prediction (Chou & Fasman, 1974), the C-terminal region of the TnC peptide has a very high propensity to form an α-helix from residue 113 to residue 119 while the CaM peptide has a high propensity for residues 102–105 only to form an α-helix. In view of the suggestions that the C-terminal α-helix is partially preformed in the apo-TnC protein and peptide (Reid et al., 1981; Reid, 1983), the difference in calcium dissociation constant of the two peptides may be due to the greater propensity of the TnC peptide to have preformed C-terminal α-helix. Partial preformation of the C-terminal α-helix may be important for calcium affinity since the $-Z$ coordinate residue is in the first turn of the C-terminal α-helix and formation of this turn will correctly position the acid residue for Ca^{2+} chelation. The possible structural differences between the two apopeptides are reflected in the CD spectra (Table I), where the $[\theta]_{222}$ for the CaM peptide is slightly less than that for the TnC peptide. Addition of Ca^{2+} to the apopeptides induces a similar amount of structure in both peptides (around 12 residues) (Table I) although the total amount of structure estimated by CD is slightly less in the CaM peptide as occurred in the apopeptides. This fact is consistent with the α-helical propensity in the peptides discussed above. The maximum amount of structure inducible in the two peptides, assumed to be that amount of structure occurring in 50% TFE, is similar for both peptides (approximately 24 residues) (Table I).

Table I: Comparative Structural Changes Induced in the STnC and CaM Peptides by Calcium and Trifluoroethanol[a]

	STnC		CaM	
	$[\theta]_{222}$ (deg·cm^2·dmol^{-1})	f_h (%)	$[\theta]_{222}$ (deg·cm^2·dmol^{-1})	f_h (%)
apopeptide	7547	27 (9)[b]	4981	18 (6)[b]
apopeptide + Ca^{2+}	17147	61 (21)	15151	54 (18)
apopeptide + TFE	20386	72 (24)	20054	71 (23)

[a] Buffers are described under Experimental Procedures. [b] Number in brackets indicates number of residues in helical structure.

The results of the comparative CD studies on these two synthetic fragments indicate that the calcium binding site III on STnC is likely of much higher affinity than its counterpart in CaM. There is also considerable support here for the Acid-Pair Hypothesis (Reid & Hodges, 1980) in that the synthetic STnC peptide with two acid pairs in the calcium binding loop region has Ca^{2+} affinity 30 times greater than that of the synthetic CaM peptide, which has no acid pairs in the calcium binding loop region. However, this difference in affinity may also be attributed to differences in the α-helical sequences or differences in the number of acid residues in chelating positions in the loop region. Studies are presently under way to examine these and other possibilities.

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SUPPLEMENTARY MATERIAL AVAILABLE

Detailed description of the synthetic protocol and HPLC

purification (10 pages). Ordering information is given on any current masthead page.

Registry No. TFE, 75-89-8; CaM(81–113), 109612-89-7; AcL^{109}CaM(81–113)amide, 109612-90-0; AcA^{98}STnC(90–123)amide, 109612-91-1; Ca, 7440-70-2.

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