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Human Brain Calmodulin: Isolation, Characterization, and Sequence of a Half-Molecule Fragment†

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ABSTRACT: A Ca^{2+}-binding protein from human brain has been purified to homogeneity and identified as residues 72–148 of calmodulin. This half-molecule fragment (CaM_{72-148}) contains 11 of calmodulin's 15 basic amino acids (including one trimethyllysine) and demonstrates a higher isoelectric point. Both tyrosines and three of eight phenylalanine residues also occur in the fragment, giving rise to a somewhat different absorption spectrum. Though it contains two of calmodulin's Ca^{2+}-binding sites, CaM_{72-148} binds only one Ca^{2+} per molecule with a dissociation constant of 17 μM. No biological activity, as judged by its inability to activate cyclic nucleotide phosphodiesterase, is observed. The sequence of amino acids is identical with that of residues 72–148 of bovine brain calmodulin [Kasai, H., Kato, Y., Isobe, T., Kawasaki, H., & Okuyama, T. (1980) *Biomed. Res. 1*, 248–264]. CaM_{72-148} is thought to arise through proteolysis, and its implications for the structure and physiological role of calmodulin are discussed.

Calmodulin is a low molecular weight, heat-stable, acidic protein that activates a variety of enzyme systems upon binding calcium [for a review, see Cheung (1980)]. It has been isolated from many species, both animal and plant, and shows a high degree of conservation of biological and physical properties. The primary structure of calmodulin from bovine brain has been completely determined (Watterson et al., 1980; Kasai et al., 1980), and partial sequences exist for bovine uterus (Grand & Perry, 1978) and rat testis (Dedman et al., 1978).

During the purification of calmodulin from human brain, another Ca^{2+}-binding protein of low molecular weight was observed. We have purified this protein to homogeneity and identified it as a fragment of calmodulin, comprising residues 72–148. The physicochemical properties of the fragment, which we call CaM_{72-148},[1] are similar to those of the parent molecule. Since calmodulin has been well characterized from

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[1] Abbreviations used: CaM_{72-148}, residues 72–148 of calmodulin; CaM_{1-71}, residues 1–71 of calmodulin; Hepes, *N*-2-(hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; $NaDodSO_4$, sodium dodecyl sulfate; PTH, phenylthiohydantoin.

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many sources, the nature of CaM_{72-148} is investigated with the aim of understanding structure–function relationships in the intact protein.

Materials and Methods

Human brains were obtained at autopsy from the Department of Pathology, University of Washington. Sephadex and Sephacryl were purchased from Pharmacia, DE-52 cellulose was from Whatman, and Chelex-100 (200–400 mesh) was from Bio-Rad laboratories. $^{45}CaCl_2$ was purchased from New England Nuclear Corp. TPCK-trypsin was obtained from Worthington, cyanogen bromide from Matheson Coleman and Bell (MCB), and trimethyl-L-lysine dioxalate hemihydrate from Bachem, Inc., Fine Chemicals. Columns for high-performance liquid chromatography (HPLC) were products of Varian, Du Pont, and Waters. Solvents used for the automated sequence analysis and HPLC were from Beckman, Pierce, and Burdick and Jackson.

Criteria of Purity. Polyacrylamide gel electrophoresis was carried out with the system of Ornstein (1964) and Davis (1964) as described by Brewer et al. (1974) in Tris–glycine buffer, pH 8.9. Electrophoresis in the presence of 0.1% sodium dodecyl sulfate ($NaDodSO_4$) was performed according to Weber & Osborne (1969) as modified by Kerrick et al. (1979). Gels were 10% throughout. Six protein standards (phosphorylase, bovine serum albumin, actin, carbonic anhydrase, troponin C, and parvalbumin) were used for molecular weight determination. Isoelectrofocusing experiments were performed in 4% gels containing 8 M urea, using 2% ampholytes (LKB-Sweden) in the pH range of 4–6 (DiSalvo et al., 1978).

Protein concentration of calmodulin was measured spectrophotometrically using the absorbancy index $A^{1\%}_{280nm} = 2.1$ (Dedman et al., 1977). Other measurements were carried out according to Lowry et al. (1951) using pure human brain calmodulin as standard.

Amino acid analyses were performed with a Durrum D502 amino acid analyzer. Samples were hydrolyzed in 6 N HCl for 24 and 48 h at 110 °C in sealed glass tubes that had been flushed with nitrogen and evacuated. Covalent phosphate was determined by the methods of Ames (1966) and Itaya & Michio (1966).

Ca^{2+}-binding measurements were carried out in 20 mM Hepes and 100 mM KCl, pH 7.4 (buffer A) that had been freed of contaminating Ca^{2+} by passage through a 2.5 × 20 cm column of Chelex-100. Stoichiometry was assessed by gel filtration on a 0.7 × 7.8 cm column of Sephadex G-25. A solution of CaM_{72-148} (about 1.5 mg/mL) in buffer A containing 10 mM $CaCl_2$ and 2.54×10^4 cpm/μL $^{45}CaCl_2$ was passed through the column, allowing separation of protein from free Ca^{2+}. Determination of protein and Ca^{2+} (by radioactive counting) concentrations in the protein peak gave the stoichiometry of binding. The dissociation constant was obtained by equilibrium dialysis. Calcium-free protein was prepared by passing the solution containing 10 mM EDTA through a 2.5 × 26 cm column of Sephadex G-25 equilibrated in buffer A. The protein that emerged (88 μM) was free of EDTA; atomic absorption measurements with a Perkin Elmer Model 290B spectrophotometer showed the presence of less than 2 μM Ca^{2+}. Dialysis experiments were performed in 1-mL circular cells divided in half by dialysis tubing with a molecular weight cutoff of 6500. To one side was added 400 μL of Ca^{2+}-free protein in buffer A, to the other 400 μL of buffer A containing varying amounts of $CaCl_2$ plus 3×10^5 cpm of $^{45}CaCl_2$. Dialysis was allowed to proceed overnight with gentle agitation at 20 °C. Partitioning of Ca^{2+} between protein and buffer compartments was assessed by scintillation counting of 40-μL aliquots from each side.

Biological activity of calmodulin was determined by its ability to activate a crude preparation of calmodulin-deficient beef brain phosphodiesterase. The enzyme was partially purified to the DE-52 stage as described by Cheung (1971). The enzymatic assay was carried out according to LaPorte et al. (1979).

Cyanogen Bromide and Tryptic Peptides. Cleavage of methionyl bonds followed the general procedure of Gross (1967). Tryptic digests were carried out in 0.1 M NH_4HCO_3, pH 8.0, at 37 °C for 3 h. Peptides were separated by gel filtration, paper electrophoresis, and HPLC. Peptide separation by HPLC was carried out with a Varian Model 5000 liquid chromatograph with a programmable gradient module. Samples were injected into columns (Varian Micro Pak MCH-10) via a Rheodyne valve fitted with a 2.5-mL sample loop. Peptides were separated by a reverse-phase system of acetonitrile.

Sequence Determination. Automated amino-terminal sequence analyses were performed with the Beckman Model 890D sequencer according to Edman & Begg (1967) as modified by either Hermodson et al. (1972) or Brauer et al. (1975). Peptides (20–100 nmol) were analyzed at approximately 95% stepwise yield in the presence of polybrene (Tarr et al., 1978). Phenylthiohydantoin derivatives of amino acids were identified by two complementary systems of reversed-phase HPLC (Bridgen et al., 1976; Ericsson et al., 1977).

Results

Purification. The purification scheme was similar to that described by Dedman et al. (1977). Four whole brains (5.35 kg) were diced, ground, and homogenized with 2 volumes of 24 mM NaH_2PO_4 and 1 mM EDTA, pH 6.5 (buffer B), for 30 s at both low and high speeds in a Waring blender. The homogenate was spun at 7120*g* for 30 min, and the supernatant was filtered through glass wool and poured into a 30-L Groen heat-treatment vessel. The temperature was raised to 90 °C with continuous stirring and maintained at that temperature for 5 min. The suspension was rapidly cooled to 20 °C and centrifuged at 7120*g* for 30 min. The supernatant was poured through glass wool, and the pellet was discarded.

Packed DE-52 (1400 mL), preequilibrated in buffer B, was added to the clear filtrate, stirred for 1.5 h, and then allowed to settle. Following aspiration of the liquid phase, the ion exchanger was poured into a Büchner funnel and washed with an additional 7 L of buffer B. The packed DE-52 was taken up in enough buffer B to form a thick slurry and poured into a 5 × 77 cm column. The elution profile is shown in Figure 1. Two pools, one for CaM_{72-148} (A) and one for calmodulin (B), were collected, based on $NaDodSO_4$–polyacrylamide gel electrophoresis. Each pool was brought to 90% saturation with ammonium sulfate at 4 °C and stirred for 1 h, after which the pH was dropped to 4.0 with 6 N HCl and the suspensions were stirred for another hour. Centrifugation at 16000*g* for 30 min followed. After the supernatants were decanted, the pellets were dissolved in a minimal amount of water, and each was dialyzed vs. 500 mL of 30 mM NaH_2PO_4, 0.1 mM EDTA, 1 mM DTT, and 0.15 M NaCl, pH 6.8 (buffer C), with one change.

The calmodulin-containing dialysate from pool B was applied to a 2.5 × 100 cm column of Sephadex G-75. A symmetrical peak eluted which showed one band on gel electrophoresis in the presence and absence of $NaDodSO_4$, and which activated a crude preparation of beef brain phosphodiesterase 12-fold. The dialysate from pool A was applied to a 5 × 88 cm column of Sephacryl S-200. Appropriate fractions were

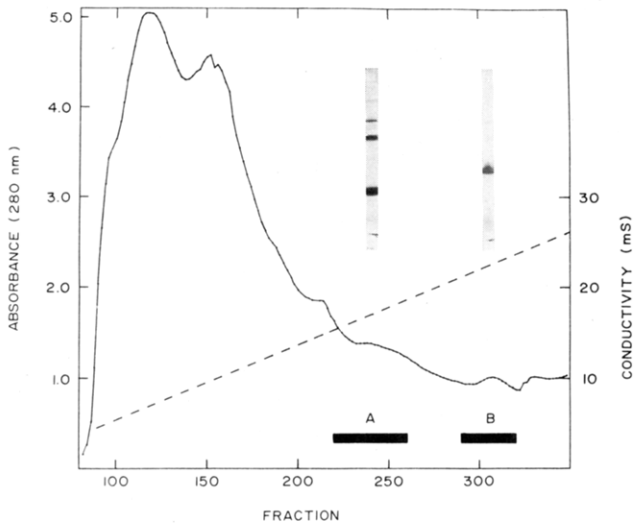

FIGURE 1: Ion-exchange chromatography of heat-treated supernatant from human brain. After batchwise adsorption onto DE-52 cellulose, the ion exchanger was poured into a 5 × 77 cm column, and a gradient of 0.1–0.4 M NaCl in buffer B was applied. The flow rate was 300 mL/h with a fraction size of 20 mL. Pooling of fractions (indicated by bars) was based on $NaDodSO_4$–polyacrylamide gels run on every fifth tube throughout the profile. Absorbance at 280 nm (—); conductivity (---).

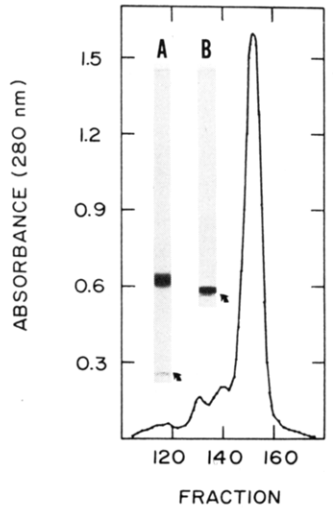

FIGURE 2: Final purification step of CaM_{72-148} on a Sephadex G-75 superfine column (1.5 × 189 cm). The column was eluted with 0.1 M NH_4HCO_3 at a flow rate of 6 mL/h, collecting 1.2 mL per tube. Fractions 146–160 were pooled. The inset shows electrophoresis patterns of CaM_{72-148} on 10% polyacrylamide gels: (A) 11-cm $NaDodSO_4$ gel, 20 μg; (B) 9-cm normal gel, 10 μg. Gels were run from top to bottom (arrow indicates dye front).

pooled, based on gel electrophoresis in the presence of $NaDodSO_4$ which demonstrated a major band of molecular weight 12 000. This material was dialyzed vs. H_2O, lyophilized, and applied to a column of Sephadex G-75 superfine (Figure 2). Material under the main peak that eluted appeared homogeneous by both normal and $NaDodSO_4$ gel electrophoresis (Figure 2, inset).

Identification of CaM_{72-148}. Amino acid analysis of the above material showed near identity with amino acids 72–148 of bovine brain calmodulin. Sequence analysis showed that the first 62 residues were identical with residues 72–133 of bovine brain calmodulin (Kasai et al., 1980). Completion of the sequence confirmed the protein's identity as residues 72–148 of calmodulin (see below).

Composition. The composition of CaM_{72-148} appears in Table I. Also presented are the amino acid composition of

Table I: Amino Acid Compositions[a] of Human Brain Calmodulin and the Fragments CaM_{1-71} and CaM_{72-148}

amino acid	CaM_{1-71}[b]	CaM_{72-148}[c]	calmodulin[d]
aspartic acid	(11)	12.0 (12)	22.6 (23)
threonine	(8)	3.9 (4)	11.3 (12)
serine	(2)	2.3 (2)	4.5 (4)
glutamic acid	(13)	15.2 (14)	28.4 (27)
proline	(2)	0.0 (0)	1.8 (2)
glycine	(6)	5.2 (5)	10.0 (11)
alanine	(5)	6.0 (6)	9.6 (11)
cysteine[e]	(0)	(0)	(0)
valine	(2)	4.8 (5)	5.4 (7)
methionine	(3)	4.6 (6)	5.6 (9)
isoleucine	(4)	3.9 (4)	7.6 (8)
leucine	(6)	3.3 (3)	9.0 (9)
tyrosine	(0)	2.0 (2)	1.9 (2)
phenylalanine	(5)	2.9 (3)	7.8 (8)
tryptophan[f]	(0)	(0)	(0)
histidine	(0)	1.0 (1)	1.2 (1)
trimethyllysine	(0)	(1)	(1)
lysine	(3)	4.9 (4)	8.3 (7)
arginine	(1)	4.8 (5)	5.8 (6)

[a] Residues/molecule by amino acid analysis after 24-h hydrolysis. Values in parentheses indicate presumptive compositions. [b] By difference from calmodulin and CaM_{72-148}. [c] Confirmed by sequence. [d] The presumptive composition has been verified by digestion of calmodulin and analysis of individual peptides (data not presented). This composition is identical with that of bovine brain according to Watterson et al. (1980) and Kasai et al. (1980). [e] Determined by performic acid oxidation. [f] Determined spectrophotometrically.

Table II: Comparison of the Properties of CaM_{72-148} and Calmodulin

property	CaM_{72-148}	calmodulin[a]
molecular weight		
gel filtration	17 000	31 000
$NaDodSO_4$–polyacrylamide gel electrophoresis	12 000	18 000[b]
sequence	8 935	16 680[b]
isoelectric point	4.4–4.5	4.3
Ca^{2+} binding		
mol of Ca^{2+} bound/mol of protein	1.0	4
K_{diss} (μM)	17	4–18
activation of phosphodiesterase	no	yes

[a] Data for bovine brain calmodulin taken from Lin et al. (1974). [b] Data from Watterson et al. (1976, 1980).

human brain calmodulin and, by difference, the presumptive composition of the first 71 amino acids of calmodulin (CaM_{1-71}). No covalently bound phosphate was detected.

Molecular weight was assessed by three methods as listed in Table II. As often observed for small acidic proteins, estimation of molecular weight by $NaDodSO_4$ gel electrophoresis and gel filtration gave anomalously high results when compared with sequence data. Isoelectrofocusing results also appear in Table II.

Spectral Properties. Ultraviolet spectra of calmodulin and CaM_{72-148} are illustrated in Figure 3. While both proteins show an absorption maximum at 276 nm, only calmodulin displays the distinct vibrational structure characteristic of phenylalanine. This is to be expected in that CaM_{72-148} has only three of the eight phenylalanyl residues in calmodulin.

Binding of Ca^{2+} to CaM_{72-148}. The stoichiometry of Ca^{2+} binding to CaM_{72-148} was measured in two separate experiments. First, the column was equilibrated in Ca^{2+}-free buffer A and the protein solution allowed to elute, giving 0.72 mol of Ca^{2+} per mol of protein in the effluent. The column was then equilibrated in buffer A containing 100 μM Ca^{2+} and

Table III: Amino Acid Compositions[a] of Peptides Obtained by Cyanogen Bromide and Tryptic Cleavage of CaM_{72-148}

	Whole	CB-1	CB-2	CB-3	CB-4	CB-5	CB-6	T-1	T-2	T-3	T-4	T-5	T-6	T-7	T-8
Residue No.	1-77	1-5	2-5	6-53	54-73	74-77	75-77	1-3	4	5-6	7-15	16-19	20-35	36-55	56-77
Figure No. & Pool		6C	6C	6A	6B	6C	6C	6F	6F	6F	6E	6F	6E	6D	6D
Amino acids															
Asx	(12)			8.1(8)	3.9(4)						2.0(2)		3.4(3)	3.4(3)	3.5(4)
Thr	(4)			2.9(3)		1.0(1)	1.0(1)				1.0(1)			2.0(2)	1.3(1)
Ser	(2)			2.3(2)							1.1(1)		1.4(1)		
Glx	(14)			10.0(9)	4.8(5)						3.3(3)	1.1(1)	1.4(1)	4.9(4)	5.1(5)
Gly	(5)			3.4(3)	2.0(2)								2.5(2)	1.5(1)	1.8(2)
Ala	(6)	1.0(1)	1.0(1)	3.5(3)	1.1(1)	1.0(1)	1.0(1)	1.0(1)				1.0(1)	2.0(2)		1.4(2)
Val	(5)			2.9(3)	1.5(2)								1.0(1)	1.7(2)	1.9(2)
Met	(6)	0.6(1)		0.1(0)		0.6(1)		0.6(1)		0.3(1)				1.3(2)	1.0(2)
Ile	(4)			1.9(2)	1.5(2)						1.0(1)		1.1(1)	1.0(1)	0.9(1)
Leu	(3)			3.1(3)									1.1(1)	1.7(2)	
Tyr	(2)			1.0(1)	0.6(1)								0.9(1)		0.7(1)
Phe	(3)			2.0(2)	1.0(1)							1.0(1)	1.1(1)		1.1(1)
His	(1)			0.8(1)										0.5(1)	
Lys	(4)	1.1(1)	1.0(1)	2.4(2)		1.0(1)	1.0(1)		1.0(1)	1.0(1)			1.0(1)		0.8(1)
Arg	(5)	1.0(1)	0.9(1)	3.0(3)	0.8(1)			0.6(1)			0.8(1)	1.0(1)	0.5(1)	0.5(1)	
Tml[b]	(1)			(1)										1.0(1)	
Hse[c]		1.2(1)	1.0(1)	1.6(2)	0.8(1)										
No. of Residues	(77)	5	4	48	20	4	3	3	1	2	9	4	16	20	22
Yield (%)		25	19	80	100	33	20	32	25	32	35	50	35	79	79

[a] Residues/molecule by amino acid analysis or (in parentheses) from the sequence (Figure 5). [b] Trimethyllysine. [c] Homoserine and its lactone.

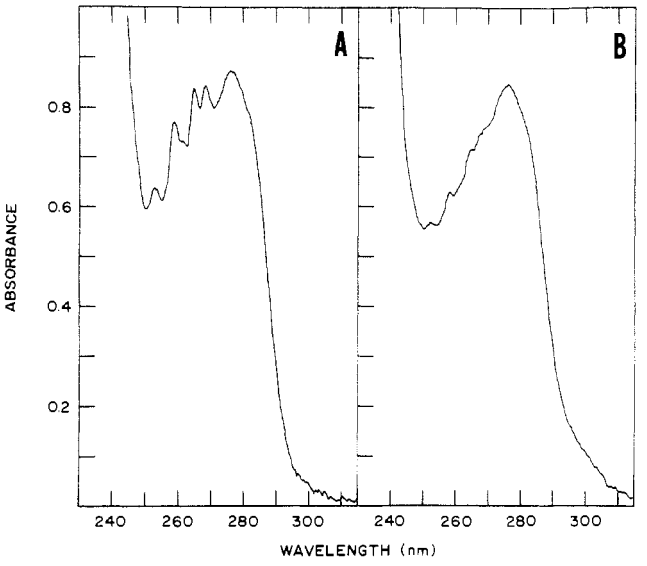

FIGURE 3: Absorption spectra of (A) calmodulin (0.43 mg/mL) and (B) CaM_{72-148} (0.17 mg/mL) from human brain. Spectra were taken in buffer A containing 1 mM $CaCl_2$.

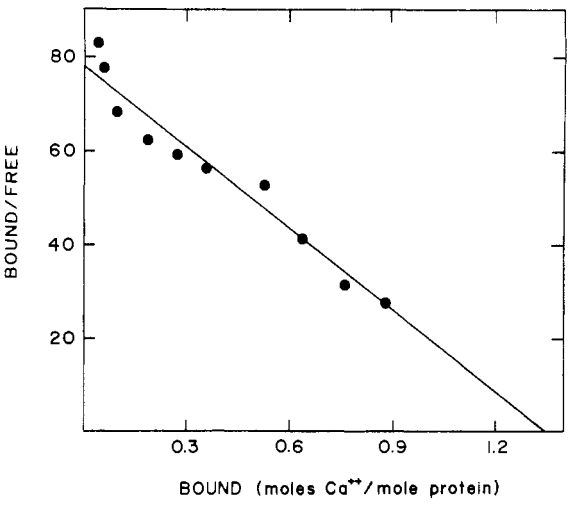

FIGURE 4: Scatchard plot of Ca^{2+} binding to CaM_{72-148}. Points are the average of three determinations.

the experiment repeated, this time revealing 1.00 mol of Ca^{2+} per mol of protein in the effluent. These results indicate a 1:1 binding of Ca^{2+} by CaM_{72-148}, therefore a single binding site. Data from equilibrium dialysis experiments were plotted according to Scatchard (Figure 4) to obtain the dissociation constant, which is given in Table II.

Biological Assay. A preparation of crude beef brain phosphodiesterase was activated to 42% of maximal stimulation by 14 ng of pure human brain calmodulin. Addition of up to 15 μg of CaM_{72-148} (1000-fold higher level) to the enzyme provided no increase over the basal activity. Moreover, the presence of CaM_{72-148} did not alter the activation of phosphodiesterase by calmodulin, indicating no competitive inhibition in this system.

Sequence Determination. The complete amino acid sequence of CaM_{72-148} is given in Figure 5. Analysis of the intact protein (70 nmol) demonstrated a 70:30 mixture of two related sequences, the minor sequence simply lacking the amino-terminal methionine. This analysis allowed positive identifications for the first 62 residues.

Cleavage of CaM_{72-148} (900 nmol) with cyanogen bromide yielded six peptides, two of which were clearly resolved by gel filtration (Figure 6, left). Four small fragments in peak C were further separated by preparative paper electrophoresis at pH 3.6. Amino acid compositions of the individual peptides are listed in Table III. Sequenator analysis of fragment CB-3 confirmed the placement of residues 6–31. Stepwise analysis of fragment CB-4 confirmed residues 54–62 and extended the sequence an additional nine amino acids. Overlapping fragments CB-5 and CB-6 represent the carboxyl-terminal portion of the molecule as they both lack homoserine.

A tryptic digest of CaM_{72-148} (400 nmol) was resolved into three peaks by gel filtration (Figure 6, right). Two peptides in peak D were separated by precipitation in 9% formic acid; peptide T-7 was soluble and T-8 insoluble in this solvent. Purification of peptides T-4 and T-6 from peak E was achieved by HPLC (Figure 7A). Peptides T-1, T-2, T-3, and T-5 were isolated from the third peak, again by HPLC (Figure 7B). Amino acid compositions of the tryptic peptides appear in Table III. The sequenator analysis of peptide T-7 confirmed residues 36–55 and allowed unambiguous identifications of

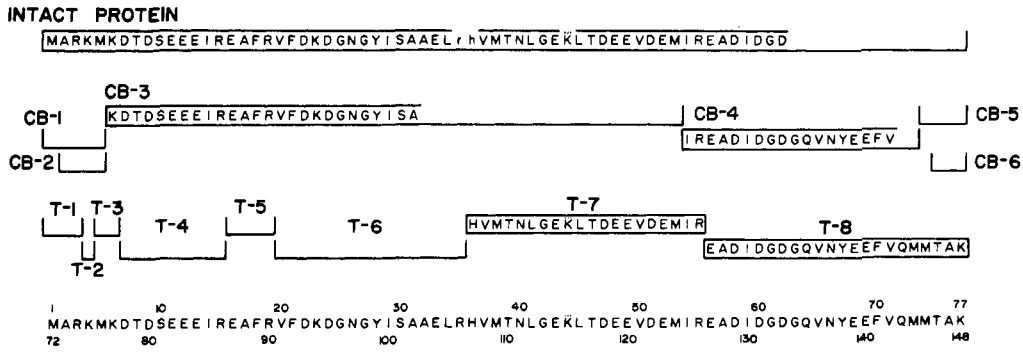

FIGURE 5: Summary proof of the sequence of CaM_{72-148}. The one-letter code within the bars designates amino acids identified after Edman degradation (capital letters) or by composition and cleavage specificity (lower case leters). The length of each bar indicates the length of that peptide. Enclosure of the top indicates a proven sequence; gaps in the upper enclosure signify portions of the sequence that were not identified. Cyanogen bromide peptides are designated by the prefix CB, tryptic peptides by the prefix T. The derived sequence of CaM_{72-148}, which appears at the bottom, is numbered both individually (1–77, above) and according to its position in calmodulin (72–148, below). One-letter amino acid abbreviations are the following: A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; K̈, trimethyllysine; L, leucine; M, methionine; N, asparagine; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; Y, tyrosine.

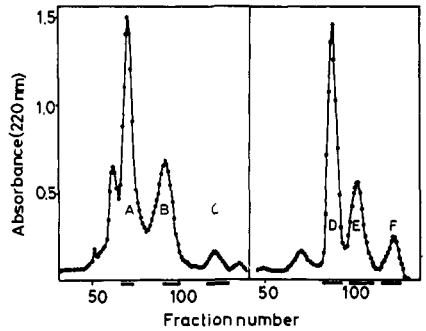

FIGURE 6: Purification of peptides from cyanogen bromide and tryptic digests of CaM_{72-148}. Left: Separation of a cyanogen bromide digest (900 nmol) on a 1.8 × 200 cm column of Sephadex G-50 superfine in 0.1 M NH_4HCO_3, pH 8.0. Fractions of 2.3 mL were collected at a flow rate of 9.4 mL/h and combined to form pools A, B, and C. Right: Separation of a tryptic digest (400 nmol) on a 1.5 × 114 cm column of Sephadex G-50 superfine in 0.1 M NH_4HCO_3, pH 8.0. Fractions of 1.3 mL were collected at a flow rate of 16 mL/h and combined as pools D, E, and F.

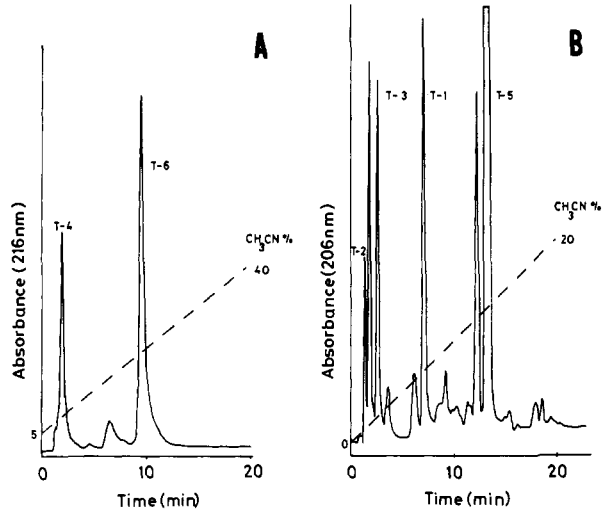

FIGURE 7: Purification of tryptic peptides by HPLC. (A) Pool E (70 nmol) was applied to a Waters μBondapak CN column (0.4 × 30 cm) and eluted by a mobile phase gradient of CH_3CN–50 mM sodium phosphate, pH 3.0, from 5:95 to 80:20 (v/v) for 40 min. (B) Pool F (50 nmol) was applied to a Varian Micro Pak MCH-10 column (0.4 × 30 cm) and eluted with the same solvent system in a gradient from 0:100 to 30:70 (v/v) for 30 min.

histidine-36 and trimethyllysine-44 (Figure 8), which had been poorly identified during analysis of the intact protein. The sequence of peptide T-8 yielded overlaps of CB-4, CB-5, and CB-6.

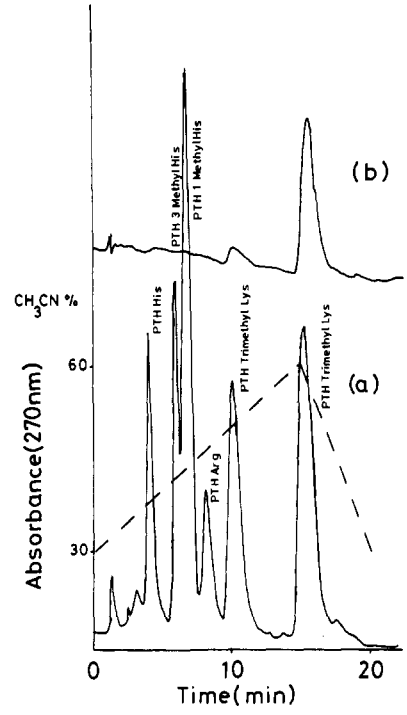

FIGURE 8: Identification of the phenylthiohydantoin (PTH) derivative of trimethyllysine. (a) PTH-derivatized standards were applied to a 0.4 × 25 cm column of Du Pont Zorbax ODS equilibrated in CH_3CN–50 mM sodium acetate (30:70 v/v). Elution was carried out with a linear gradient from the initial solvent to a 60:40 (v/v) mixture of CH_3CN–50 mM sodium acetate at a flow rate of 2.0 mL/min. (b) Elution of the PTH derivative from cycle 9 of the sequential degradation of peptide T-7.

Discussion

This paper reports the simultaneous purification from human brain of calmodulin and a fragment of calmodulin (residues 72–148). The yield of purified CaM_{72-148} was in excess of 10 mg/kg of starting material. This compares with a typical yield of 50 mg/kg for calmodulin, which we have observed in other preparations from this source.

The origin of CaM_{72-148} would appear to be from proteolytic degradation of whole calmodulin, with the break occurring between methionine-71 and methionine-72 (70%) or between methionine-72 and alanine-73 (30%). There remains the question of when the proteolysis takes place. All brains were obtained within 24-h post-mortem and stored at −20 °C until preparation (1 day to 2 weeks). Degradation could occur (a) in vivo, (b) in the immediate post-mortem period, (c) during

storage at −20 °C, or (d) in the course of purification. Nonetheless, it is surprising to find such large quantities of a homogeneous peptide that is presumed to be a proteolytic fragment. An intriguing though speculative interpretation is that calmodulin contains an exposed region near the center of the molecule that is particularly vulnerable to proteolytic attack. In fact, Walsh & Stevens (1978) have shown that methionine residues 71, 72, and 76 are available to solvent molecules in the presence of Ca^{2+}. Furthermore, the selective oxidation of these residues abolished the interaction of calmodulin with phosphodiesterase. Since this region of the protein appears to be vital to its activity, a single clip in vivo would release two biologically inactive fragments, thus terminating its physiological role. This explanation provides a rapid method for cellular turnover of calmodulin that accounts for the 77-residue peptide we observe.

The physiochemical properties of CaM_{72-148} are quite similar to those of calmodulin. The key to the differences is the amino acid composition data, listed in Table I. CaM_{72-148} contains 11 of calmodulin's 15 basic residues; thus, its isoelectric point is higher (Table II). The presence of both tyrosines and three of eight phenylalanines gives CaM_{72-148} an absorbancy index approximately twice that of calmodulin. However, the higher tyrosine to phenylalanine ratio obliterates the fine structure due to phenylalanine that is present in the intact molecule's spectrum (Figure 3). The molecular weight of CaM_{72-148} is lower than that of calmodulin when assessed by gel filtration, $NaDodSO_4$–polyacrylamide gel electrophoresis, and sequence analysis.

The Ca^{2+}-binding parameters are of particular interest. Amino acids 72–148 comprise slightly over half of calmodulin and should contain two of the four binding sites for Ca^{2+}; yet CaM_{72-148} binds only one Ca^{2+} per molecule. It is not known which of the two sites remains functional. One possible scenario is that the site nearest the amino terminus, normally tucked away in the interior of calmodulin, is now exposed to a different, aqueous environment in which it assumes a nonfunctional conformation. The carboxy-terminal site remains relatively unaffected and continues to bind Ca^{2+}. The dissociation constant indicates that this Ca^{2+} is perhaps the most weakly bound of the four, and there is recent work suggesting that the carboxyl-terminal binding domain has the weakest affinity for Ca^{2+} (Haiech et al., 1981). Alternatively, the relatively weak binding of Ca^{2+} may reflect important changes in protein conformation as a result of the molecule's new anatomy.

No activation of phosphodiesterase is detected in CaM_{72-148} despite its ability to bind one Ca^{2+}. This is consistent with the work of Walsh et al. (1977) in which limited tryptic digestion of calmodulin produced fragments tentatively identified by amino acid analysis as residues 1–77 and residues 78–148. Neither fragment could activate phosphodiesterase, though the latter peptide underwent a conformational change in the presence of Ca^{2+} and was capable of Ca^{2+}-dependent interaction with troponin I. Under different conditions of digestion, fragments consisting of residues 1–106 and 107–148 were generated. The former was reported to have limited activity toward phosphodiesterase (over 200-fold less active than native calmodulin) as well as interacting with troponin I. In combination with these previously reported results, our data suggest that the calmodulin molecule must be intact to retain biological activity.

The completed sequence of CaM_{72-148} has allowed its unequivocal identification. The placement of amino acids is identical with residues 72–148 of bovine brain calmodulin as determined by Kasai et al. (1980). There are two differences with the sequence of Watterson et al. (1980), at residues 129 and 135. In both cases, only the assignment of an amide or acid group is different. The amino acid sequence of human brain calmodulin has recently been completed, and further discussion of its significance will appear elsewhere (T. Sasagawa, L. H. Ericsson, K. A. Walsh, W. E. Schreiber, E. H. Fischer, and K. Titani, unpublished experiments).

In summary, the fortuitous discovery of a fragment of calmodulin has allowed an unusual view into structure–function relationships in this increasingly important protein.

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Purification and Pharmacological Properties of Eight Sea Anemone Toxins from *Anemonia sulcata*, *Anthopleura xanthogrammica*, *Stoichactis giganteus*, and *Actinodendron plumosum*†

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ABSTRACT: Eight different polypeptide toxins from sea anemones of four different origins (*Anemonia sulcata*, *Anthopleura xanthogrammica*, *Stoichactis giganteus*, and *Actinodendron plumosum*) have been studied. Three of these toxins are new; the purification procedure for the five other ones has been improved. Sea anemone toxins were assayed (i) for their toxicity to crabs and mice, (ii) for their affinity for the specific sea anemone toxin receptor situated on the Na^+ channels of rat brain synaptosomes, and (iii) for their capacity to increase, in synergy with veratridine, the rate of $^{22}Na^+$ entry into neuroblastoma cells via the Na^+ channel. Some of the toxins are more active on crustaceans, whereas others are more toxic to mammals. A very good correlation exists between the toxic activity to mice, the affinity of the toxin for the Na^+ channel in rat brain synaptosomes, and the stimulating effect on $^{22}Na^+$ uptake by neuroblastoma cells. The observation has also been made that the most cationic toxins are also the most active on mammals and the least active on crustaceans. Toxicities (LD_{50}) to mice of the most active sea anemone toxins and of the most active scorpion toxins are similar, and sea anemone toxins at high enough concentrations prevent binding of scorpion toxins to their receptor. However, scorpion toxins have affinities for the Na^+ channel which are ~60 times higher than those found for the most active sea anemone toxins. Three sea anemone toxins appear to be more interesting than toxin II from *A. sulcata* (the "classical" sea anemone toxin) for studies of the Na^+ channel structure and mechanism when the source of the channel is of a mammalian origin. Two of these three toxins can be radiolabeled with iodine while retaining their toxic activity; they appear to be useful tools for future biochemical studies of the Na^+ channel.

Sea anemone toxins are among the most interesting tools for analysis of the properties of the voltage-dependent Na^+ channel. The sequences of five of these polypeptide toxins are currently available (Wunderer et al., 1976a; Wunderer & Eulitz, 1978; Béress et al., 1977; Martinez et al., 1977; Tanaka et al., 1977; Norton et al., 1978) and the structure–function relationships of toxin II from *Anemonia sulcata* (AS_{II})[1] have been recently established (Barhanin et al., 1981). Sea anemone toxins selectively slow down the closing (inactivation) of the Na^+ channel. They do not interfere with the binding of tetrodotoxin or saxitoxin near the selectivity filter of the Na^+ channel (Romey et al., 1976; Jacques et al., 1978). Their binding site is different from those of other toxins which also act on the gating system of the Na^+ channel like veratridine, batrachotoxin, aconitine, grayanotoxin, and pyrethroids (Vincent et al., 1980). Sea anemone toxins have been shown to interact with a large variety of excitable membranes including myelinated and nonmyelinated axons (Rathmayer & Béress, 1976; Romey et al., 1976; Bergman et al., 1976), neuronal cells in culture (Jacques et al., 1978), cardiac and skeletal muscle cells in culture (De Barry et al., 1977; Romey et al., 1980), and nerve terminals (Abita et al., 1977).

We describe in this paper the purification of eight toxins obtained from four different sea anemone species. The activity of each toxin was followed by measuring its toxicity on crabs and mice, its affinity for the specific sea anemone toxin receptor that has been characterized in rat brain synaptosomes (Vincent et al., 1980), and its stimulating effect on the rate of $^{22}Na^+$ uptake by neuroblastoma cells in culture.

Materials and Methods

Materials. Sephadex G-50 fine, G-25 fine, and G-10, SP-Sephadex C-25, CM-Sephadex C-25, and QAE-Sephadex A-25 were purchased from Pharmacia Fine Chemicals. Veratridine was obtained from Aldrich Chemical Co. Dulbecco's modified Eagle's medium and fetal calf serum were from Gibco. Buffers and salts were analytical grade products

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[1] Abbreviations used: AS_I, AS_{II}, AS_{III}, and AS_V, toxins I, II, III, and V from the sea anemone *Anemonia sulcata*, respectively; $[^{125}I]AS_{II}$, radiolabeled monoiodo derivative of AS_{II}; AX_I and AX_{II}, toxins I and II from the sea anemone *Anthopleura xanthogrammica*, respectively; SG_I and SG_{II}, toxins I and II from the sea anemone *Stoichactis giganteus*, respectively; AP_I, toxin I from the sea anemone *Actinodendron plumosum*; AaH_{II}, toxin II from the scorpion *Androctonus australis* Hector; $[^{125}I]AaH_{II}$, radiolabeled iodo derivative of AaH_{II}; MU and CU, mouse and crab unit, respectively; CM, carboxymethyl; Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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