Complete Amino Acid Sequence of Human Brain Calmodulin†

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ABSTRACT: The complete amino acid sequence of calmodulin from human brain has been determined by using peptides derived from digests with trypsin, *Staphylococcus aureus* V8 protease, and cyanogen bromide. The peptides were purified by means of reversed-phase high-performance liquid chromatography and analyzed with a sequenator. The protein contains 148 amino acid residues and has a molecular weight of 16792. As in other calmodulins, the amino-terminal residue of the protein is blocked by an acetyl group, and a trimethyllysine residue is located at position 115. The only difference between this sequence and those fully determined in other species is the assignment of amide groups.

Calmodulin is a low molecular weight, heat-stable, and acidic protein which reversibly forms a complex with calcium ions. The calmodulin–Ca^{2+} complex stimulates the activity of a number of enzymes (Kakiuchi et al., 1970; Cheung, 1971; Brostrom et al., 1975; Jarrett & Penniston, 1978; Dabrowska et al., 1978; Cohen et al., 1978). It has been isolated from many species, and a high degree of conservation by biological and physical properties has been found (Cheung, 1980; Klee et al., 1980; Means & Dedman, 1980).

The complete amino acid sequences of bovine brain (Watterson et al., 1980; Kasai et al., 1980) and rabbit skeletal muscle calmodulin (Grand et al., 1981) have been determined, as well as partial sequences for those from bovine uterus (Grand & Perry, 1978) and rat testis (Dedman et al., 1978). However, several discrepancies have been observed in the assignment of amides. In a previous report (Schreiber et al., 1981), we have detailed the isolation of human brain calmodulin and described the physicochemical properties and amino acid sequence of a half-molecule fragment, CaM_{72-148}.[1] In the present paper, the amino acid sequence of the whole molecule is presented and compared with the reported structures of calmodulins from other sources.

Materials and Methods

Calmodulin was purified from human brain as described by Schreiber et al. (1981). TPCK-trypsin, α-chymotrypsin, and pepsin were purchased from Worthington. Carboxypeptidase Y was a gift from Dr. M. Ottesen (Carlsberg Laboratory, Copenhagen). *Staphylococcus aureus* V8 protease was obtained from Miles. Columns for reversed-phase HPLC were obtained from the indicated sources: Micropak MCH-10 (Varian), μBondapak C18 (Waters), Zorbax ODS (Du Pont). Phenacyl bromide and 18-crown-6 were purchased from Aldrich.

Trimethyllysine was determined in acid hydrolysates with a Dionex D-500II analyzer according to the method of Van Eldik et al. (1980). The amino-terminal blocking group was identified as the phenacyl ester by the method of Durst et al. (1975), but using reversed-phase HPLC.

Protein was citraconylated according to the method of Atassi et al. (1972). Tryptic digestion of the citraconylated protein (37 °C, pH 8.8 for 1 h) was terminated by the addition of lima bean trypsin inhibitor. These peptides were separated by gel filtration at pH 8.8 to retain the citraconylated forms. Subsequent decitraconylation was carried out in 9% formic acid for 3 h at 37 °C. Methods of enzymatic digestion with trypsin, chymotrypsin, pepsin, *S. aureus* V8 protease, and carboxypeptidase Y have been described by Koide et al. (1978). In most cases of peptide separation by HPLC, the mobile phase was 0.1% trifluoroacetic acid, and the mobile phase modifier was acetonitrile containing 0.07% trifluoroacetic acid (Dunlap et al., 1978; Mahoney & Hermodson, 1980). The concentration of acetonitrile was increased linearly (1%/min) during 50 min at a flow rate of 2 mL/min.

Automated sequence analyses were performed with a Beckman sequencer (Model 890) according to Edman & Begg (1967) as modified by Brauer et al. (1975), and using Polybrene (Tarr et al., 1978). Phenylthiohydantoin (Pth) derivatives of amino acids were identified by two complementary systems of reversed-phase HPLC (Ericsson et al., 1977; Hermann et al., 1978). Table V (see paragraph at end of paper regarding supplementary material) summarizes these automated Edman degradations, and Figure 10 (supplementary material) illustrates the quantitative data of one representative peptide. In each analysis, identifications of Pth's were judged to be proven if a single Pth appearing in a given cycle clearly rose above the background of the previous cycle and dropped in the next cycle. We regard these observations as semiquantitative in nature and hesitate to document all of the apparent yields. In these analyses, appropriate subtraction of background Pth's and correction for incomplete reaction in each cycle introduce rather arbitrary adjustments of yields which are more realistically expressed by the qualitative judgments in Table V (supplementary material) than by the seemingly more precise quantitations in Figure 10 (supplementary material).

Other methods and materials have been previously described (Schreiber et al., 1981).

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[1] Abbreviations: CaM_{72-148}, residues 72–148 of calmodulin; HPLC, high-performance liquid chromatography, Pth, phenylthiohydantoin.

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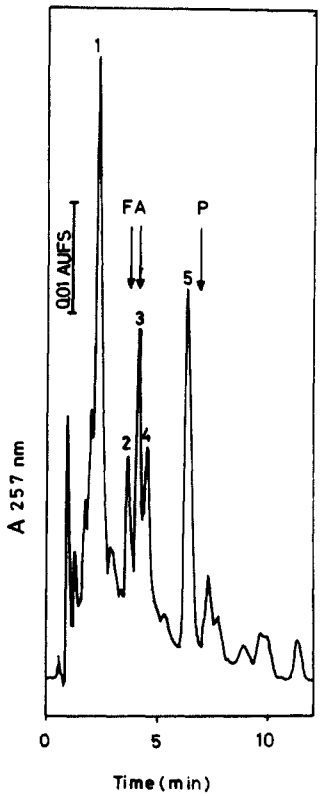

FIGURE 1: Identification of amino-terminal blocking group. Human calmodulin (10 nmol) was subjected to esterification after acid hydrolysis, and an aliquot (2.5 nmol) was analyzed on a column of Zorbax ODS. The mobile phase was 40% acetonitrile, and the flow rate was 2 mL/min. Phenacyl esters of formic, acetic, and propionic acids eluted at the positions indicated by F, A, and P, respectively. Peak 3 corresponded to the phenacyl ester of acetic acid (1 nmol) and peak 5 to α-dibromoacetophenone. Peaks 1, 2, and 4 were not identified but were observed in reagent blanks in the absence of calmodulin.

Results

Identification of Amino-Terminal Blocking Group. Human calmodulin (50 nmol) was subjected to 10 cycles of Edman degradation without release of Pth derivatives. However, 10 nmol of whole protein yielded 4 nmol of the phenacyl ester of acetic acid by the method of Durst et al. (1975) (Figure 1), indicating that the amino terminus is blocked by α-N-acetylation as in other calmodulins.

Products of Arginyl Cleavage. A tryptic digest of citraconylated calmodulin (1.8 μmol) was resolved into seven fractions by gel filtration (Figure 2a). Fraction III contained two peptides, acid-soluble Tc1 and acid-insoluble Tc2. They were separated by precipitation of Tc2 in 9% formic acid. Fraction IV contained four peptides, one of which (Tc8) was insoluble in 9% formic acid and precipitated from the mixture. The soluble peptides Tc4, Tc6, and Tc7 were separated by reversed-phase HPLC (Figure 2b). Fractions V and VI contained Tc6 and Tc9, respectively. Fraction VII contained two peptides (Tc3 and Tc5) which were separated by HPLC (Figure 2c). The amino acid compositions of these peptides are shown in Table I. In summary, seven major peptides were isolated, only one of which (Tc8) lacked arginine. Two minor peptides (Tc3 and Tc9) were also isolated.

Five of these peptides (Tc3, Tc4, Tc5, Tc7, and Tc8) were sequenced through their carboxyl-terminal argininyl residues as shown in Table V (supplementary material) and Figure 3. The sequence analysis of Tc2 yielded positive identifications of 30 out of 37 degradation cycles [Table V, Figures 3 and 10 (supplementary material)]. Tentative identifications in cycles 23, 25, and 33–37 were confirmed by separation and sequencing of peptides obtained by subdigestion of Tc2 with *S. aureus* V8 protease. Similarly, the balance of Tc6 was determined in a chymotryptic peptide Tc6-C3. Peptide Tc1 was resistant to Edman degradation, but a tryptic digest of Tc1 was resolved into three peptides (Tc1-T1, Tc1-T2, and Tc1-T3), and the latter two were completely sequenced (Figure 3). Since peptide Tc1-T3 contained the carboxyl-terminal arginine of Tc1 and peptide Tc1-T2 contained a free amino-terminal group, the three subpeptides were aligned as in Figure 3 with blocked peptide Tc1-T1 at the amino terminus.

Peptide Tc1-T1 (40 nmol) was further digested with pepsin, and the products were resolved into six peptides, which were subjected to sequence analysis (Figure 3). The carboxyl-terminal lysine of Tc1-T1-P4 placed it at the carboxyl terminus of Tc1-T1. Only Tc1-T1-P1 was blocked, which placed it at the amino terminus of Tc1-T1 (Figure 3). The sequence of

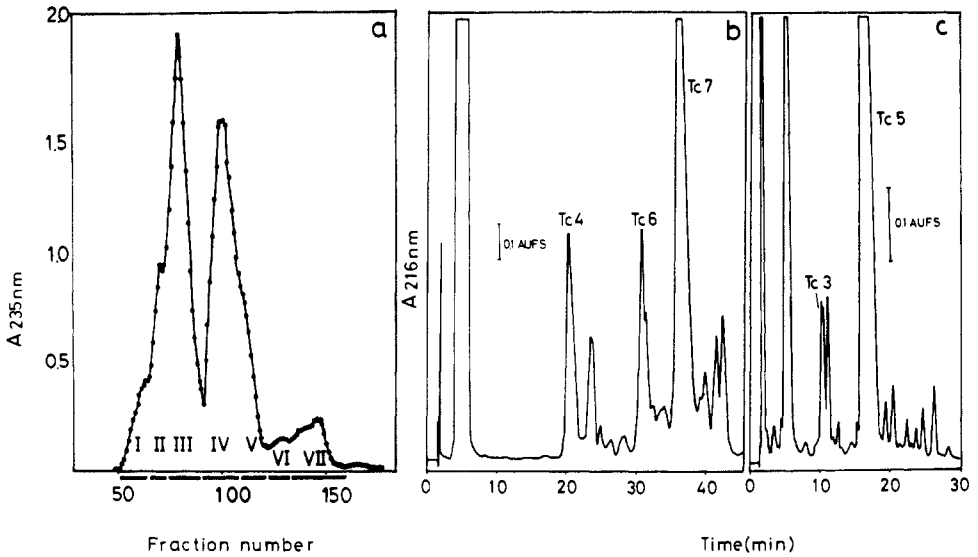

FIGURE 2: Separation of peptides obtained by arginine cleavage of calmodulin. (a) Separation on Sephadex G-50. A tryptic digest of citraconylated calmodulin (1.8 μmol) was applied to a Sephadex G-50 SF column (1.8 × 200 cm) and eluted with 0.1 M ammonium bicarbonate at a flow rate of 9.4 mL/h. Fractions of 2.2 mL were collected. The elution was monitored by UV absorption (235 nm). The fractions indicated by the horizontal bars were collected. (b) Subfractionation of fraction IV (9% formic acid soluble fraction) by reversed-phase HPLC. Sample (450 nmol) was loaded on a Micropak MCH-10 column. The elution conditions are described under Materials and Methods. (c) Subfractionation of fraction VII by reversed-phase HPLC. Sample (900 nmol) was loaded on a Micropak MCH-10 column.

Table I: Amino Acid Compositions[a] of Peptides Obtained by Arginine Cleavage of Calmodulin

residue no. / pool no. (Figure 2a)	whole 1-148	Tc1 1-37 III	Tc2 38-74 III	Tc3 72-74 VII	Tc4 75-86 IV	Tc5 87-90 VII	Tc6 91-106 V	Tc7 107-126 IV	Tc8 127-148 IV	Tc9 145-148 VI
Asx	22.6 (23)	4.3 (4)	7.0 (7)		1.9 (2)		3.0 (3)	2.9 (3)	4.0 (4)	
Thr	11.3 (12)	4.5 (5)	2.8 (3)		1.0 (1)			1.8 (2)	1.3 (1)	
Ser	4.5 (4)	1.2 (1)	1.0 (1)		0.9 (1)		1.0 (1)			1.0 (1)
Glx	28.4 (27)	7.0 (7)	7.4 (6)		3.0 (3)	1.1 (1)	1.7 (1)	4.0 (4)	5.1 (5)	
Pro	1.8 (2)		2.0 (2)							
Gly	10.0 (11)	2.9 (3)	3.3 (3)				1.9 (2)	1.1 (1)	2.0 (2)	
Ala	9.6 (11)	2.8 (3)	2.9 (3)	1.0 (1)		1.0 (1)	1.8 (2)		1.8 (2)	1.0 (1)
Val	5.4 (7)	0.9 (1)	1.3 (1)				1.0 (1)	1.7 (2)	1.8 (2)	
Met	5.6 (9)	0.8 (1)	1.2 (3)	0.8 (1)	0 (1)			1.0 (2)	1.7 (2)	0.4 (1)
Ile	7.6 (8)	1.9 (2)	2.1 (2)		0.9 (1)		1.0 (1)	1.0 (1)	1.1 (1)	
Leu	9.0 (9)	3.0 (3)	3.5 (3)				1.0 (1)	1.4 (2)		
Tyr	1.9 (2)						0.8 (1)		0.8 (1)	
Phe	7.8 (8)	2.8 (3)	2.6 (2)			1.0 (1)	0.9 (1)		1.1 (1)	
His	1.2 (1)							0.8 (1)		
Lys	7.6 (7)	2.9 (3)			1.9 (2)		1.1 (1)		0.9 (1)	1.0 (1)
Arg	5.8 (6)	0.8 (1)	0.9 (1)	1.0 (1)	0.9 (1)	1.0 (1)	0.9 (1)	0.9 (1)		
TML[b]	1.2 (1)							1.0 (1)		
no. of residues	148	37	37	3	12	4	16	20	22	4
yield (%)		85	73	24	100	83	100	68	43	1

[a] Residues/molecules by amino acid analysis or (in parentheses) from the sequence (Figure 3). [b] Trimethyllysine.

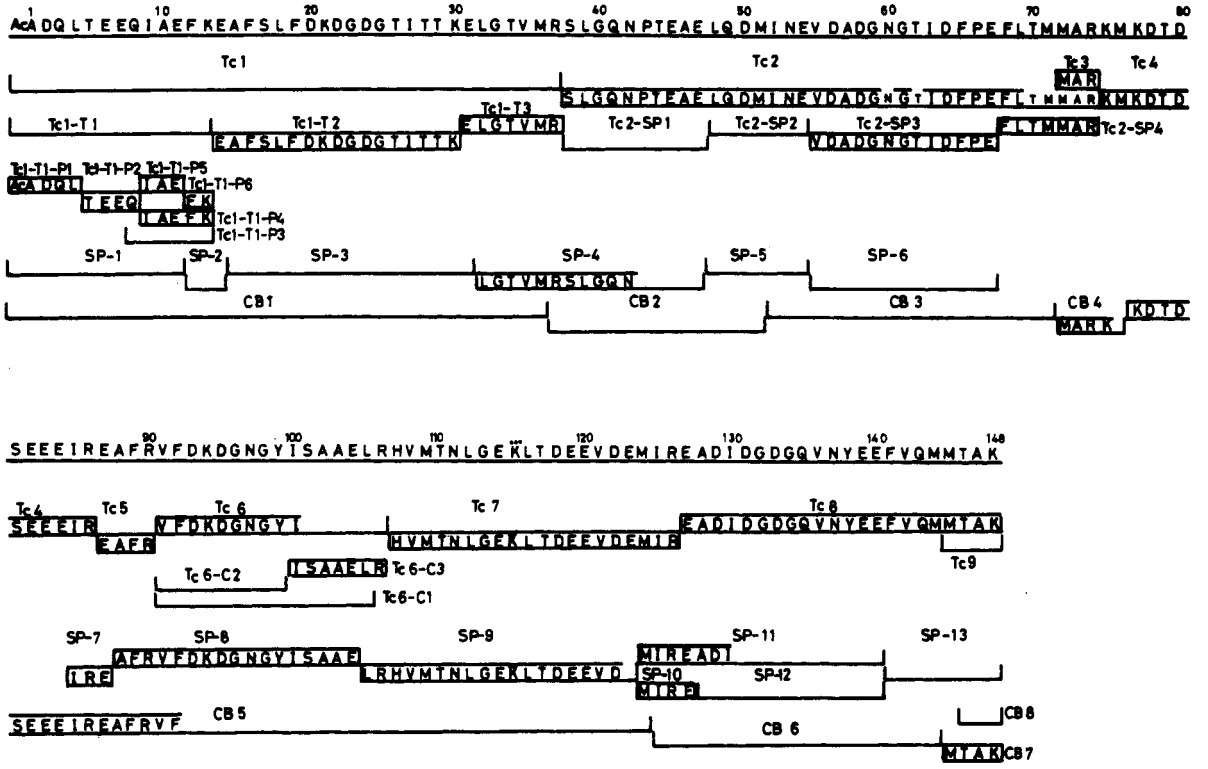

FIGURE 3: Summary proof of the sequence of human brain calmodulin. The one-letter code (see Figure 6) within bars designates amino acids identified after Edman degradation (capital letters) or by composition and cleavage specificity (small letters). The length of each bar indicates the proportional length of that peptide. Enclosure of the top indicates a proven sequence; gaps in the upper enclosure signify portions of that sequence that were not identified. Tryptic, chymotryptic, peptic, and cyanogen bromide peptides are designated by the prefix T, C, P, and CB, respectively. The peptides cleaved at a glutamyl residue are designated by the prefix SP. Tryptic peptides after citraconylation are designated by the prefix Tc.

Tc1-T1-P1 was determined from its carboxyl terminus by using carboxypeptidase Y (Figure 4). Leucine, glutamine, and aspartic acid were sequentially released, but not alanine. The amino-terminal blocked alanine was purified by HPLC from the 4-h digest and showed behavior identical with that of *N*-acetylalanine on a Waters C18 column, indicating that the amino terminus of human calmodulin is α-*N*-acetylalanine. Thus, the structure of *N*-acetyl-Ala-Asp-Gln-Leu was assigned to Tc1-T1-P1.

Peptides Tc3 and Tc9 corresponded to the carboxyl-terminal region of Tc2 and Tc8, respectively (Figure 3). Apparently they arose by tryptic cleavage of Met–Met bonds, as observed in other calmodulins (Watterson et al., 1980; Kasai et al., 1980).

Alignment of Tc Peptides. Peptide Tc1 was placed at the blocked amino terminus of the protein. Peptide Tc8, the only major Tc peptide which lacked arginine, was placed at the carboxyl terminus. The remaining Tc peptides were aligned

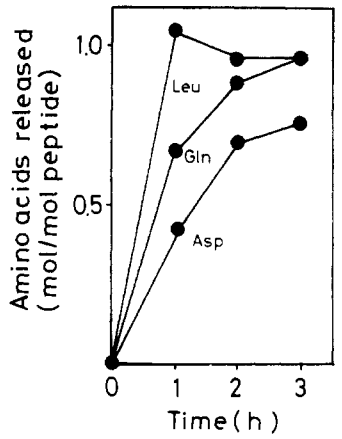

FIGURE 4: Time course of carboxypeptidase Y digestion of peptide Tc1-T1-P1. Aliquots of the carboxypeptidase digest at 1, 2, and 3 h were subjected to amino acid analysis without acid hydrolysis.

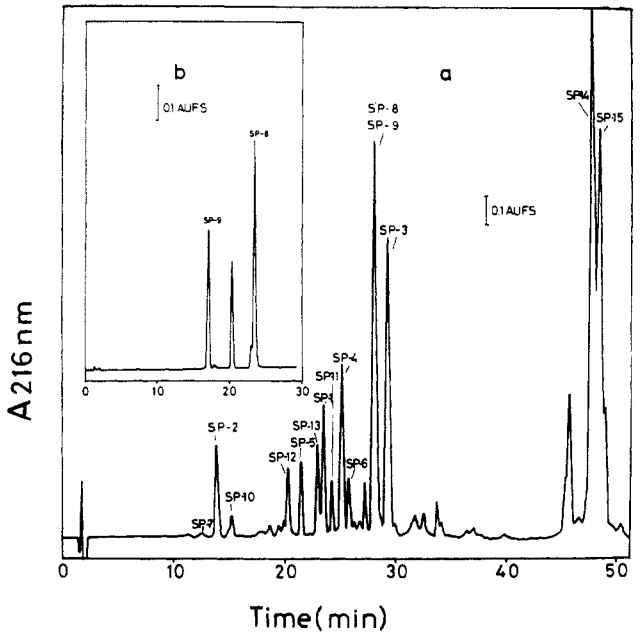

FIGURE 5: (a) Separation of peptides obtained by cleavage of calmodulin at glutamyl residues with *S. aureus* V8 protease. The digest (80 nmol) was separated on a column of μBondapak C18. The mobile phase was 0.1% trifluoroacetic acid as described under Materials and Methods. (b) Separation of SP-9 from SP-8 on the same column, but using 0.005 M sodium phosphate (pH 6.5) as the mobile phase.

with peptides obtained by cleavage at glutamyl residues (Figure 3). After digestion of the protein (80 nmol) with staphylococcal protease, the mixture was fractionated by HPLC (Figure 5). Six peptides (SP-4, SP-7, SP-8, SP-9, SP-10, and SP-11) were found by sequence analysis (Figure 3) to provide overlaps for Tc1/Tc2 and for the five peptides from Tc4 through Tc8. The other peptides from this digest corresponded in composition to those expected from the established interior portions of the tryptic peptides (Figure 3). Amino acid compositions of SP-14 and SP-15 corresponded to large products of incomplete cleavage (residues 55–148 and 12–104). The alignments of Tc2/Tc4 and of Tc4/Tc5 were established by the sequences of CB4 and CB5, peptides derived by fragmentation of calmodulin with cyanogen bromide.

Discussion

The complete amino acid sequence of calmodulin from human brain is established as shown in Figure 6. The protein

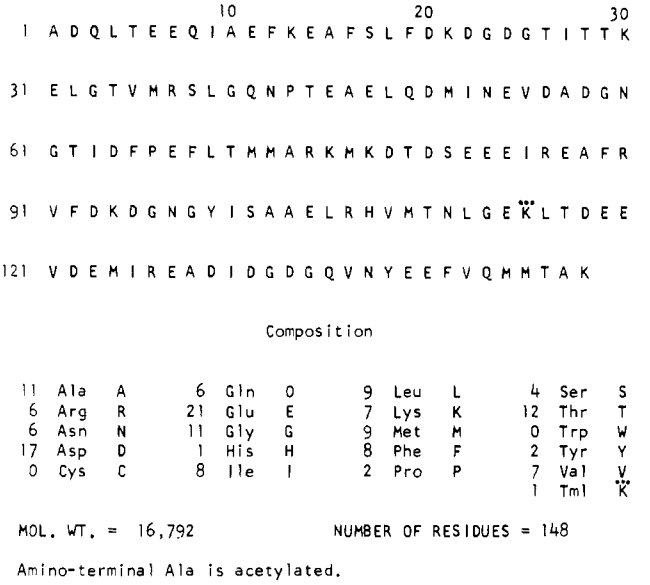

FIGURE 6: Amino acid sequence of human brain calmodulin. The amino acid composition beneath the sequence defines the one-letter code notations.

is composed of a single polypeptide chain of 148 residues. As with calmodulins from other organisms, it contains trimethyllysine at residue 115 and an acetylated amino terminus. As expected, the sequence of residues 72–148 is identical with that of CaM_{72-148} (Schreiber et al., 1981). This strongly suggests that CaM_{72-148} is derived by proteolytic degradation of whole calmodulin. The particular bond cleaved, between methionyl residues 71 and 72, was also cleaved during the tryptic digestion in the present experiments, indicating unusual lability.

Comparison of human brain calmodulin with those from other sources shows near identity. The only differences are in amide assignments (Figure 7) except at residue 60 in the partially completed sequence of the rat testis protein by Dedman et al. (1978). Although these differences could be due to mistakes in identification of the Pth derivatives or different degrees of deamidation during the purification of the proteins or their peptides, they could represent real differences at the gene level or unrecognized in vivo amidation/deamidation processes. In our study and in that by Grand et al. (1981), the Pth derivatives were identified by reversed-phase HPLC, whereas Watterson et al. (1980) used gas chromatography and Kasai et al. (1980) used normal phase HPLC. All these methods should distinguish between the acid and amide forms, so mistakes are unlikely. Grand et al. (1981) discussed the possibility of selective deamidation during purification. An alternative possibility is suggested by the pattern of discrepancies. Of the 50 sites identified as either acids or amides, differences among the species are now documented at 11 separate loci. Of these 11, 6 fall within the putative calcium-binding regions postulated by Tufty & Kretsinger (1975). On could, therefore, speculate that affinity of calmodulin for calcium may be adjusted in vivo by unrecognized amidation and deamidation processes. It should be emphasized that there is no direct data to support this speculation.

The available data indicate that the amino acid sequence of calmodulin has been almost perfectly conserved through evolution from rabbit (Grand et al., 1981) to human. This adds strength to the argument that there are few degrees of freedom for change of this calcium-binding protein which would be compatible with maintenance of its ability to interact with its cellular partners.

	2	3	6	7	8	11	14	20	22	24	31	41	42	45	47	49	50	53	54	56	58	60	64	67	78
Human brain[a]	D	Q	E	E	Q	E	E	D	D	D	E	Q	N	E	E	Q	D	N	E	D	D	N	D	E	D
Bovine brain[b]										N															
Bovine brain[c]																						D			
Bovine uterus[d]																									
Rat testis[e]		E											D					D				A			
Rabbit skeletal muscle[f]																									

	80	82	83	84	87	93	95	97	104	111	114	118	119	120	122	123	127	129	131	133	135	137	139	140	143
Human brain[a]	D	E	E	E	E	D	D	N	E	N	E	D	E	E	D	E	E	D	D	D	Q	N	E	E	Q
Bovine brain[b]																		N			E				
Bovine brain[c]																									
Bovine uterus[d]																		N							
Rat testis[e]								D		D								N				D			E
Rabbit skeletal muscle[f]																		N							

[a]Present work, [b]Watterson et al.(1980), [c]Kasai et al.(1980), [d]Grand and Perry (1978), [e]Dedman et al.(1978) and [f]Grand et al.(1981).

FIGURE 7: Differences in the assignment of amides among various calmodulins. Numbers correspond to residue numbers. The underlined regions correspond to putative calcium-binding sites. Only differences are shown.

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Supplementary Material Available

Four tables and three figures giving descriptions of the purification, amino acid compositions, and automated Edman degradations of peptides and subpeptides (8 pages). Ordering information is given on any current masthead page.

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