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Structural and Functional Properties of Calmodulin from the Eukaryotic Microorganism *Dictyostelium discoideum*†

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ABSTRACT: Calmodulin was purified from the eukaryotic microorganism *Dictyostelium discoideum* and characterized in terms of its nearly complete primary structure and quantitative activator activity. The strategy for amino acid sequence analysis took advantage of the highly conserved structure of calmodulin and employed a new procedure for limited cleavage of calmodulin that uses a protease from mouse submaxillary gland. Fourteen amino acid sequence differences between *Dictyostelium* and bovine calmodulin were identified unequivocally, as well as an unmethylated lysine at residue 115 instead of N^{ϵ},N^{ϵ},N^{ϵ}-trimethyllysine. Seven of the amino acid substitutions in *Dictyostelium* calmodulin are novel in that the residues at these positions are invariant in all calmodulin sequences previously examined, most notably an additional residue at the carboxy terminus. Comparison of the *Dictyostelium* calmodulin sequence with other calmodulin sequences shows that the region with the greatest extended sequence identity includes parts of the first and second structural domains and the interdomain region between domains 1 and 2. *Dictyostelium* calmodulin activated bovine brain cyclic nucleotide phosphodiesterase in a manner indistinguishable from that of bovine brain calmodulin. However, *Dictyostelium* calmodulin activated pea NAD kinase to a maximal level 4.6-fold greater than that produced by bovine brain calmodulin. This functional difference demonstrates the potential biological importance of the limited number of amino acid sequence differences between *Dictyostelium* calmodulin and other calmodulins and provides further insight into the structure, function, and evolution of the calmodulin family of proteins.

Calmodulin is a calcium binding protein that has been found in all eukaryotic cells examined in detail. It has multiple in vitro biochemical activities and has a highly conserved primary structure [for recent reviews, see Klee & Vanaman (1982) and Burgess et al. (1983)]. Complete amino acid sequences are available for calmodulin from bovine brain (Watterson et al., 1980a, 1984) and human brain (Sasagawa et al., 1982). Nearly complete sequences have been reported for calmodulin from rabbit skeletal muscle (Grand et al., 1981), rat testis (Dedman et al., 1978), scallop muscle (Toda et al., 1981), *Metridium senile* (Takagi et al., 1980), *Tetrahymena pyriformis* (Yazawa et al., 1981), *Renilla reniformis* (Jamieson et al., 1980), and spinach leaf (Burgess et al., 1983; Watterson et al., 1983). In addition, Lagace et al. (1983) and Putkey et al. (1983) inferred amino acid sequences for eel and chicken calmodulin from the nucleotide sequences of multiple, overlapping cDNAs. Other than a single amino acid substitution in eel calmodulin (Lagace et al., 1983), the amino acid sequences of all vertebrate calmodulins characterized to date appear to be identical. Among invertebrates, higher plants, and protozoa, there are several amino acid substitutions relative to the vertebrate protein (Burgess et al., 1983; Van Eldik et al., 1982).

The calmodulin molecule contains four structural domains that are homologous to each other and to the four domains of troponin C (Watterson et al., 1980a). These structural domains contain regions homologous to the portions of parvalbumin that form a calcium binding structure (Kretsinger, 1980). The first and second domains of calmodulin are more closely related respectively to domains 3 and 4 than are other pairs of domains (Watterson et al., 1980a). This intramolecular sequence homology is the basis of hypotheses that calmodulin may have evolved by gene duplication of a one- or two-domain precursor (Vanaman et al., 1977; Erickson et al., 1980). Accordingly, calmodulin from phylogenetically earlier species such as *Tetrahymena pyriformis* and *Dictyostelium discoideum* might be more similar to the postulated precursors than calmodulin from phylogenetically later species. An inherent assumption of such phylogenetic analyses of structure and function is that regions of extended amino acid sequence identity among calmodulins from closely related and

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distant species reflect functionally important domains that have been conserved during evolution due to selective pressures.

As part of a phylogenetic analysis of calmodulin structure and function, the eukaryotic microorganism *D. discoideum* offers several advantages. Calcium has been shown to affect cell aggregation (Mason et al., 1971) and cytoplasmic contractility (Condeelis & Taylor, 1977; Hellewell & Taylor, 1979) in this organism, and the interaction of calcium with several proteins implicated in cell motility has been analyzed [reviewed by Spudich & Spudich (1982)]. The *Dictyostelium* life cycle includes a growth phase in which the haploid unicellular amoebas carry out various motile activities including cytokinesis, chemotaxis, phagocytosis, and pinocytosis. Starvation triggers a developmental phase in which individual amoebae produce and respond chemotactically to pulses of cAMP[1] [reviewed by Devreotes (1982)]. The role of calmodulin in *Dictyostelium* is not known. However, by extrapolation from its in vitro activities with extracts of higher organisms (Klee & Vanaman, 1982), calmodulin might be involved in the regulation of motility and development in *Dictyostelium*.

The purification and characterization of *Dictyostelium* calmodulin are fundamental to understanding the molecular basis of calcium and calmodulin action in *Dictyostelium*. Because the eukaryotic protists are evolutionarily distant from the vertebrates (Stanier et al., 1963), elucidation of the primary structure of *Dictyostelium* calmodulin is important to the deciphering of the molecular evolution of this highly conserved protein and its relatives (Goodman, 1980). An earlier report (Bazari & Clarke, 1981) described the isolation and several properties of calmodulin from vegetative amoebas of *D. discoideum*. This paper describes the further characterization, including amino acid sequence analysis and quantiative activator activity, of *Dictyostelium* calmodulin that has been purified further by chromatography on [(phenylethyl)silanyl]silica or phenyl-Sepharose.

Materials and Methods

Purification of Dictyostelium Calmodulin. Amoebae of *Dictyostelium discoideum*, strain AX3, were grown on HL5 medium (Clarke et al., 1980) and harvested at a density of (2–3) × 10^7 cells/mL. The cells were collected and broken as previously described (Clarke et al., 1980) except that benzamidine (100 μg/mL) was included in the lysis medium. Calmodulin was purified by two methods, which yielded protein that was indistinguishable by elution profile, electrophoretic mobility, amino acid composition, and peptide maps. In the first method, calmodulin was purified as previously described (Bazari & Clarke, 1981) and then subjected to reverse-phase chromatography on [(phenylethyl)silanyl]silica according to the procedure of Klee et al. (1981) as modified by Marshak et al. (1981). Following chromatography on Bio-Gel P-60 (Clarke et al., 1980), the protein was dialyzed against water, lyophilized, and redissolved in solvent A (10 mM potassium phosphate, pH 6.1, 0.5 mM EGTA). The sample was applied to a column (0.78 × 30 cm) of [(phenylethyl)silanyl]silica (Waters, μBondapak phenyl) which was equilibrated in 80% (v/v) solvent A and 20% (v/v) solvent B (acetonitrile). Nonadsorbed material was eluted with 20% (v/v) solvent B at a flow rate of 2 mL/min. Calmodulin was eluted by changing the composition of the mobile phase to 28% (v/v) solvent B. The effluent was collected and rotary evaporated to remove acetonitrile and to reduce the volume by ~40%. The protein solution was then dialyzed exhaustively against 10 mM ammonium bicarbonate and lyophilized.

In the second method, the original protocol (Bazari & Clarke, 1981) was followed through chromatography on DE-52. Fractions containing calmodulin were pooled and dialyzed against 20 volumes of buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM 2-mercaptoethanol, and 0.1 mM $CaCl_2$. The pool was then subjected to chromatography on phenyl-Sepharose CL-4B (Pharmacia) as described by Gopalakrishna & Anderson (1982). For an initial quantity of 100 g of cells (wet wt), a 1.5-mL column was used. This purification yielded approximately 0.7 mg of calmodulin/100 g of cells.

Chromatography on either phenyl-Sepharose or [(phenylethyl)silanyl]silica removed from the calmodulin contaminating material that was not detectable by polyacrylamide gel electrophoresis. The amino acid composition of this material accounted for the differences between the amino acid composition of *Dictyostelium* calmodulin previously reported (Bazari & Clarke, 1981) and that reported here.

Amino Acid Analysis. Samples for amino acid analysis were hydrolyzed in vacuo in 0.5 mL of 6 N HCl (Ultrex, J. T. Baker) with a small crystal of phenol for 24 h at 115 °C. Following incubation, the samples were rotary evaporated to dryness and dissolved in sample dilution buffer (Pierce) containing 0.2 N sodium citrate, pH 2.20, 2% (w/v) thiodiglycol, and 0.1% (w/v) phenol. The samples were analyzed on an LKB 4400 instrument interfaced with a Hewlett-Packard 3356/3357 laboratory automation system. Analyses of samples for N^{ϵ},N^{ϵ},N^{ϵ}-trimethyllysine were performed with an isothermal program as described by Van Eldik et al. (1980) as modified by Schleicher et al. (1984). Cysteine was determined as cysteic acid following performic acid oxidation according to the procedure described by Hirs (1967).

Several peptides were isolated in small amounts (0.7–8 nmol) by preparative high-performance liquid chromatography on (octadecylsilanyl)silica. The amino acid compositions of these peptides contained levels of serine and glycine that were higher than expected. In control experiments, the chromatographic analyses were performed with the same solvents and gradient programs but without samples. The amino acid compositions of fractions collected from these control effluents contained serine (0.5–1.5 nmol), glycine (1.0–2.4 nmol), and variable amounts of aspartic acid, glutamic acid, and alanine.

The amino acids in peptide fraction R2-A were analyzed without hydrolysis by a modification of the method described by Pfeifer et al. (1983). The fraction was evaporated under vacuum to dryness, redissolved in 0.30 mL of 0.01 M ammonium bicarbonate, and evaporated under vacuum to dryness. The residue was dissolved in 0.03 mL of buffer containing 0.2 N sodium citrate, pH 2.20, 2% (w/v) thiodiglycol, and 0.1% (w/v) phenol. An aliquot (0.01 mL) of the sample was mixed with an equal volume of buffer containing 1 M potassium borate, pH 10.4, 0.18% (w/v) Brij 35, 0.037 M *o*-phthalaldehyde, and 5% (v/v) 2-mercaptoethanol. The sample was mixed for 30 s at room temperature and applied to a column (0.29 × 15 cm) of (octadecylsilanyl)silica (Waters Resolve column) equilibrated in 90% (v/v) solvent A [0.05 M sodium acetate and 0.05 M sodium phosphate, pH 7.5, containing 2% (v/v) tetrahydrofuran and 2% (v/v) methanol] and 10% solvent B [65% (v/v) methanol in water] at room temperature, with a flow rate of 1.50 mL/min. The following programmed steps were used, with a Waters 680 controller to elute the

[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; AMP, adenosine 5′-monophosphate; Pth, phenylthiohydantoin; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; Tris, tris(hydroxymethyl)aminomethane.

amino acid derivatives: 0 min, % B 10; 14 min, % B 50, curve 7 (concave upward); 23 min, % B 100, curve 6 (linear), 24 min, % B 100. The fluorescence of the effluent was monitored with an LKB 4460 fluorescence detector, interfaced with a Hewlett-Packard 3357 laboratory automation system.

Protease Digestion. Dictyostelium calmodulin was digested with mouse submaxillary gland protease (Boehringer), which has a relative specificity for arginyl peptide bonds as substrates (Schenkein et al., 1977). Calmodulin (17.6 nmol) was dissolved in 0.1 M ammonium bicarbonate and 1 mM EGTA, pH 7.8, to a protein concentration of 0.6 mg/mL. An aliquot (0.011 mL) of mouse submaxillary gland protease (0.67 mg/mL in water) was added to the calmodulin at a 1:50 (w/w) ratio. Following incubation for 2 h at 37 °C, a second aliquot (0.011 mL) of protease was added, and the solution was incubated for an additional 14–18 h at 37 °C. The final ratio of protease to calmodulin was 1:25 (w/w). Following incubation, the sample was rotary evaporated to dryness and dissolved in 50 mM ammonium bicarbonate.

Separation of Peptides from Submaxillary Gland Protease Digest. Peptides from the digestion of *Dictyostelium* calmodulin with mouse submaxillary gland protease were separated by high-performance liquid chromatography with a procedure similar to that previously described (Watterson et al., 1980b), as described in Figure 3. The absorbance of the effluent was monitored at 215 nm, and the detector signal was digitized and collected with a Hewlett-Packard 3356 laboratory automation system. Fractions of the effluent corresponding to peaks of absorbance were collected (Figure 3), evaporated to dryness, and dissolved in 10 mM ammonium bicarbonate.

Cyanogen Bromide Digestion. Dictyostelium calmodulin was digested with cyanogen bromide according to the procedure of Gross (1967). Calmodulin (119 nmol) was dissolved in 0.5 mL of 70% (v/v) formic acid, and 0.0714 mL of 6 M CNBr in acetonitrile was added. This gave a 400-fold molar excess of CNBr over methionines. The solution was incubated for 22 h at room temperature and then rotary evaporated to dryness. The residue was redissolved in 1.0 mL of 0.05 M ammonium bicarbonate and incubated at room temperature for 3 h. The solution was centrifuged for 2 min at 9000*g* at room temperature. The supernatant (0.96 mL) was fractionated by high-performance liquid chromatography as described in Figure 6 (supplementary material; see paragraph at end of paper regarding supplementary material).

Trypsin Subdigestion of Peptides CB11, R2, and R3. Peptides CB11 (42.9 nmol), R2 (4.6 nmol), and R3 (8.2 nmol) were further digested with trypsin at an enzyme to peptide ratio of 1:50 (w/w), and the resulting peptides were separated by high-performance liquid chromatography as described in Figures 7, 9, and 10 (supplementary material).

Thermolysin Subdigestion of Peptide CB11-C. Peptide CB11-C (8.64 nmol) was further digested with thermolysin (Calbiochem) at an enzyme to peptide ratio of 1:30 (w/w) for 32 h at 37 °C. The peptide products of the digest were separated by high-performance liquid chromatography as described in Figure 8 (supplementary material).

Nomenclature of Peptides. Peptides were assigned numbers or letters in correspondence to their order of elution in chromatographic separations. Peptides isolated from digestion with submaxillary gland protease are numbered R1 through R4, and peptides isolated from digestion with cyanogen bromide are numbered CB1 through CB12. Peptides isolated from subdigestions with trypsin are assigned letters (e.g., R2-A through R2-F and CB11-A through CB11-C). The peptides isolated from the thermolysin digestion of peptide CB11-C are numbered TH-1 through TH-4.

Automated Sequence Determination of Peptides. An aliquot of each peptide (0.3–6.4 nmol) was subjected to automated amino acid sequencer determination with an Applied Biosystems 470A instrument under the standard program supplied by the manufacturer and with procedures described by Hewick et al. (1981) with the following modifications. Prior to application of the peptide, filters were treated with 0.029 mL of polybrene (60 mg/mL in water) and subjected to six cycles of treatment with the sequencing reagents and solvents. Peptides, dissolved in 10 mM ammonium bicarbonate, were applied to the treated filters in aliquots of 0.030 mL and dried. Fractions from the sequencer were dried and dissolved in 0.060 mL of methanol (Fisher, HPLC grade) for analysis.

Pth-amino acids were identified by high-performance liquid chromatography on a column (0.41 × 25 cm) containing (octadecylsilanyl)silica (Zorbax ODS, Du Pont) operated at 60 °C according to a modification of the procedure of Zimmerman et al. (1977). The elution solvents consisted of 50 mM sodium acetate, pH 5.0 (solvent A), and acetonitrile (solvent B). The Pth-amino acids were eluted at a flow rate of 1.5 mL/min, and the absorbance of the effluent was monitored at 254 nm. The following programmed steps were used: 0 min, % B 10; 8 min, % B 40; 12 min, % B 40; 13 min, % B 45; 16 min, % B 60. Pth-Ser and Pth-Thr were also detected by the absorbance of the effluent at 313 nm monitored from 10.4 to 11.5 min following injection.

Secondary identification of Pth-Phe and Pth-Lys was performed by using chromatography on the (octadecylsilanyl)-silica column and solvent system described above, equilibrated and eluted isocratically at 42% B. In some cases, Pth-Phe, Pth-Lys, and Pth-Arg were also identified by chromatography on a column (0.41 × 25 cm) of [(cyanopropyl)silanyl]silica (Zorbax CN, Du Pont) operated at room temperature. The elution solvents consisted of 20 mM sodium acetate, pH 5.8 (solvent A), and acetonitrile (solvent B), the flow rate was 1.5 mL/min, and the absorbance of the effluent was monitored at 269 nm. The following programmed steps were used: 0 min, % B 25; 4 min, % B 25; 8 min, % B 35; 12 min, % B 35; 13 min, % B 50; 15 min, % B 50.

NAD Kinase Activity. NAD kinase was extracted and partially purified by modifications of previously described procedures (Muto & Miyachi, 1977; Jarret et al., 1982). Two-week-old pea seedlings (110 g) were homogenized in 300 mL of 25 mM triethanolamine–HCl, pH 7.5, 0.5% (w/v) polyvinylpolypyrrolidone, and 1 mM phenylmethanesulfonyl fluoride. The homogenate was squeezed through cheesecloth and centrifuged at 12000*g* for 30 min. NAD kinase in the supernatant was purified through the protamine sulfate and polyethylene glycol precipitation steps as described by Muto and Miyachi (1977). The precipitate obtained from the polyethylene glycol step was suspended in 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 3 mM $MgCl_2$, and 0.1 mM EGTA and was passed through a column (4 × 1 cm) of DEAE-Sephadex A-25. The effluent contained NAD kinase activity that was completely dependent upon calcium and exogenous calmodulin for activity. The partially purified NAD kinase was stored at −80 °C until use.

NAD kinase activity was measured by using the method described by Wang & Kaplan (1954) as modified by Muto & Miyachi (1977). NADP production was measured by using the procedure of Apps (1970).

Other Procedures. The activation of bovine brain cyclic nucleotide phosphodiesterase activity was measured by the procedure described by Watterson et al. (1980c). Bovine brain

Table I: Amino Acid Compositions of *Dictyostelium* Calmodulin and Peptides[a] from Cyanogen Bromide Digestion

	calmodulin								
	mol per 17018 g	by sequence	CB1	CB2	CB3	CB6	CB7	CB9	CB11
aspartic acid	26.2	24		1.0 (1)	2.2 (2)	5.0 (5)	6.0 (6)	8.3 (7)	3.3 (3)
threonine	9.2	10			0.9 (1)		1.3 (1)	3.3 (4)	3.6 (4)
serine	6.2	7			0.9 (1)		0.4 (0)	1.8 (2)	3.4 (4)
glutamic acid	28.1	27			4.2 (4)	3.1 (3)	3.5 (2)	10.1 (10)	8.0 (8)
proline	1.8	2			1.2 (1)		0.7 (1)		
glycine	11.4	11			1.1 (1)	2.1 (2)	2.1 (2)	3.4 (3)	3.4 (3)
alanine	10.4	10	1.0 (1)		1.0 (1)	1.0 (1)	1.5 (1)	3.1 (3)	3.1 (3)
cysteine[b]	0	0							
valine	8.0	8		0.5 (1)		1.7 (2)	1.3 (1)	2.9 (3)	1.0 (1)
methionine	8.7	9	*c* (1)		*c* (1)	*c* (2)	*c* (2)	*c* (2)	*c* (1)
isoleucine	7.8	8		0.6 (1)		0.8 (1)	1.7 (2)	1.9 (2)	1.8 (2)
leucine	10.2	10			2.0 (2)	0.9 (1)	1.2 (1)	2.7 (3)	2.8 (3)
tyrosine	2.0	2				0.9 (1)		0.7 (1)	
phenylalanine	7.8	8				0.9 (1)	1.7 (2)	2.0 (2)	2.8 (3)
histidine	1.0	1						0.9 (1)	
trimethyllysine	0	0							
lysine	8.0	8	1.0 (1)			1.0 (1)	0.6 (0)	2.7 (3)	2.8 (3)
arginine	6.1	6	0.9 (1)	1.0 (1)	1.0 (1)	0.8 (1)		1.5 (2)	
tryptophan	0[d]	0							
yield (%)			45	51	36	36	35	14	40
residue no.[e]			73–76	146–149	37–51	125–145	52–72	77–124	1–36

[a] Peptides refer to fractions from the chromatographic separation shown in Figure 6 (supplementary material). Amino acid compositions of peptides are molar ratios with the number of residues expected from the amino acid sequence shown in parentheses. [b] Determined as cysteic acid after performic acid oxidation. [c] Qualitative detection of homoserine. [d] Excluded spectrophotometrically. [e] Residue number refers to those in Figure 1.

calmodulin standard was isolated according to the procedure of Watterson et al. (1980b) as modified by Marshak et al. (1981). All protein concentrations were determined by amino acid analysis.

Results

Calmodulin was purified from *Dictyostelium* as described under Materials and Methods. The amino acid composition of *Dictyostelium* calmodulin, shown in Table I, is similar to that of calmodulin from vertebrate and higher plant species. *Dictyostelium* calmodulin is rich in acidic residues and contains 1 mol of histidine and 2 mol of proline/mol of protein. Like vertebrate calmodulin, *Dictyostelium* calmodulin contains 2 mol of tyrosine/mol of protein and is devoid of cysteine. However, *Dictyostelium* calmodulin does not contain N^{ϵ},N^{ϵ},N^{ϵ}-trimethyllysine, as do bovine and spinach calmodulins. In addition, the threonine to serine ratio of *Dictyostelium* calmodulin is 1.4, in contrast to those of spinach (2.3) and bovine (3.0) calmodulins. These results suggest that *Dictyostelium* calmodulin is a unique chemical structure but do not provide information about the structural relatedness of *Dictyostelium* calmodulin to other calmodulins.

Strategy for Amino Acid Sequence Analysis. Amino acid sequence analysis of *Dictyostelium* calmodulin was performed in order to further establish the homogeneity of the protein preparation and to compare the primary structure with those of other calmodulins. Sequence analysis of the intact protein indicated that it was blocked at the amino-terminal residue. Therefore, the strategy for sequence analysis employed peptides isolated from a cyanogen bromide digest of the protein and peptides isolated from proteolytic subdigestions of the amino-terminal cyanogen bromide peptide. The linear sequence of cyanogen bromide peptides was established by amino acid sequence analysis of peptides isolated from a digest of the protein with mouse submaxillary gland protease. The results of these studies allowed the assignment of 146 positions of a total of 151 amino acid residues per molecule (Figure 1). Of

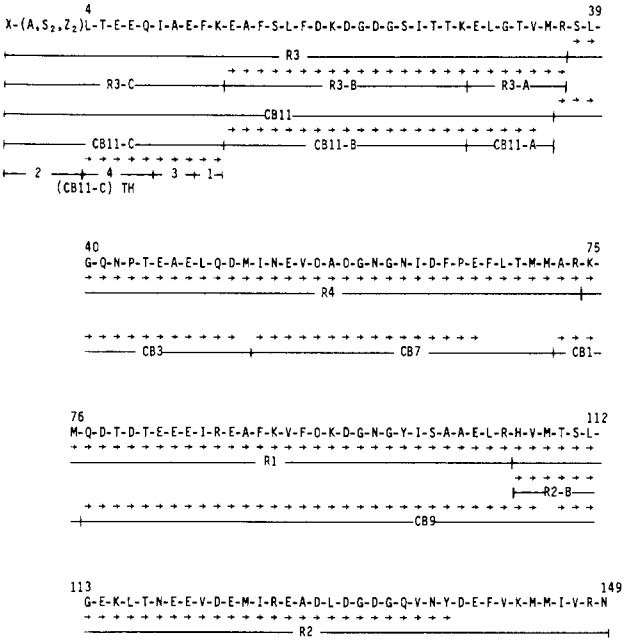

FIGURE 1: Primary structure of calmodulin from *D. discoideum*. The peptides used for amino acid sequence analysis of calmodulin are shown. The residues are numbered according to the amino acid sequence of bovine brain calmodulin (Watterson et al., 1980a). The peptides are labeled as follows: cyanogen bromide digestion, CB1–CB11; submaxillary gland protease digestion, R1–R4; trypsin subdigestions, A–F (e.g., CB11-A, R2-F); thermolysin subdigestion of CB11-C, (CB11-C) TH-1–TH-4. The symbol (→) indicates automated Edman degradation of the amino acid residue.

these 146 assignments, 119 were established from peptides with overlapping sequences, and 27 residues in the amino terminus of the protein (residues 4–30) were aligned with the homologous sequences found in bovine brain calmodulin (Watterson et al., 1980a).

Table II: Amino Acid Compositions[a] of Peptides[b] Isolated from Trypsin Subdigestion of Peptide CB11 and Thermolysin Subdigestion of Peptide CB11-C

	CB11-A	CB11-B	CB11-C	TH-1	TH-2	TH-3	TH-4
aspartic acid		3.2 (3)					
threonine	0.9 (1)	1.8 (2)	1.0 (1)				1.0 (1)
serine		1.8 (2)	1.9 (2)		1.8 (2)		
glutamic acid	1.2 (1)	1.3 (1)	6.1 (6)		2.1 (2)	1.1 (1)	3.3 (3)
proline							
glycine	1.1 (1)	2.0 (2)					
alanine		1.1 (1)	2.1 (2)		1.0 (1)	1.0 (1)	
valine	1.1 (1)						
methionine	c (1)						
isoleucine		0.9 (1)	0.9 (1)			0.9 (1)	
leucine	1.0 (1)	1.0 (1)	1.0 (1)				1.0 (1)
tyrosine							
phenylalanine		2.0 (2)	1.0 (1)	1.0 (1)			
histidine							
lysine		2.0 (2)	1.1 (1)	1.0 (1)			
arginine							
yield (%)	69	70	22	80	78	82	68
residue no.[d]	31–36	14–30	1–13	12–13	1–3[e]	9–11	4–8

[a] Molar ratios are given with the number of residues expected from the sequence shown in parentheses. [b] Peptides refer to fractions from the chromatographic separations shown in Figures 7 and 8 (supplementary material). [c] Qualitative detection of homoserine. [d] Residue number refers to those in Figure 1. [e] The amino acid sequence of peptide TH-2 was not assigned but is assumed to include residues 1–3.

Sequences of Peptides from Cyanogen Bromide Digestion. *Dictyostelium* calmodulin was digested with cyanogen bromide, and the products were separated by high-performance liquid chromatography ((Figure 6, supplementary material). The amino acid compositions and recoveries of the peptides used for sequence analysis are shown in Table I. The recovery of peptide CB9 is low due to partial cleavage at the methionyl–threonyl bond at positions 109 and 110 as previously described (Watterson et al., 1980a). The sequences of peptides CB1, -2, -3, -6, -7, and -9 are summarized in Figure 1, and the automated sequencer determinations are given in Tables 9 and 10 (supplementary material). Peptide CB2 was placed at the carboxy terminus of the protein because it was the only peptide from the cyanogen bromide digest that did not contain homoserine.

Peptide CB11 had an amino acid composition similar to residues 1–36 of bovine brain calmodulin (Figure 2). This peptide was digested with trypsin, and the products were separated by high-performance liquid chromatography (Figure 7, supplementary material). The amino acid compositions and recoveries of the peptides are shown in Table II. Peptide CB11-A contained homoserine and was placed, therefore, as the carboxy-terminal tryptic peptide of CB11. Sequence analysis (Table 11, supplementary material) yielded residues 31–36. The amino acid composition and amino acid sequence of peptide CB11-B corresponded to residues 14–30 by homology with the bovine brain sequence (Figure 2). The amino acid composition of peptide CB11-C resembled residues 1–13 of bovine brain calmodulin (Figure 2).

For further characterization, peptide CB11-C was subdigested with thermolysin, the products were isolated by high-performance liquid chromatography (Figure 8, supplementary material), and their amino acid compositions are shown in Table II. The amino acid compositions of peptides TH-1, TH-3, and TH-4 accounted for residues 4–13 of *Dictyostelium* calmodulin, and the sequences of these peptides [Table 11 (supplementary material) and Figure 1] were identical with residues 4–13 of the bovine protein (Figure 2). The amino acid composition of the remaining peptide, TH-2, was similar to residues 1–3 of bovine brain calmodulin but contained no aspartic acid, an additional glutamic acid, and two additional serine residues (Table II).

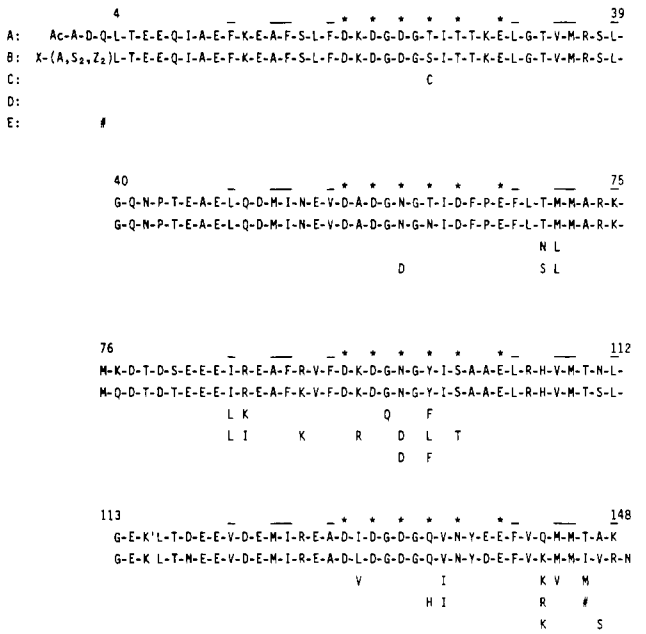

FIGURE 2: Sequence alignment of calmodulins. Row A shows the complete amino acid sequence of bovine brain calmodulin (Watterson et al., 1980a,b), and row B shows the alignment of the sequence data obtained for *Dictyostelium* caomodulin. The known amino acid sequence differences from bovine brain calmodulin are shown for (row C) spinach leaf calmodulin (Burgess et al., 1983; Watterson et al., 1983a), (row D) *Tetrahymena* calmodulin (Yazawa et al., 1981), and (row E) *Renilla* calmodulin (Jamieson et al., 1980). Asterisks (*) indicate positions of proposed calcium-ligating structures, and bars (—) indicate residues proposed to be on the internal aspect of α-helices (Kretsinger, 1980). The single-letter abbreviations for the amino acids are as follows: A, alanine; B, aspartic acid or asparagine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; X, unidentified; Z, glutamic acid or glutamine. K′ indicates N^{ϵ},N^{ϵ},N^{ϵ}-trimethyllysine. The symbol (#) indicates a gap in the sequence introduced to give maximal alignment.

Sequences of Peptides from Submaxillary Gland Protease Digest. Peptides from calmodulin treated with mouse submaxillary gland protease were isolated by reverse-phase high-performance liquid chromatography, as shown in Figure 3. The sequences of these peptides are summarized in Figure

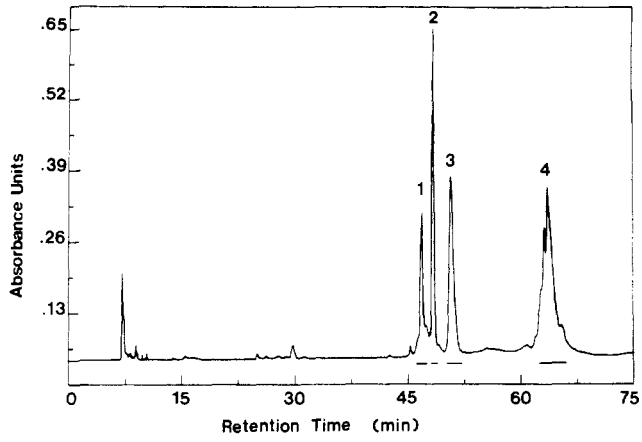

FIGURE 3: Separation by high-performance liquid chromatography of peptides generated from 12.6 nmol of *Dictyostelium* calmodulin treated with mouse submaxillary gland protease. The ordinate shows the absorbance of the effluent at 215 nm, and the abscissa shows the retention time in minutes. An aliquot of the digest (12.6 nmol in 1.15 mL) was applied to a column (0.94 × 25 cm) of (octadecylsilanyl)silica (Whatman ODS-3) equilibrated in 95% (v/v) 0.01 N HCl (solvent A) and 5% (v/v) acetonitrile (solvent B) at 2 mL/min and 30 °C on a Hewlett-Packard 1084B liquid chromatograph. The following programmed steps were used to elute peptides from the column; 5 min, % B 5; 5.5 min, % B 10; 10 min, % B 10; 20 min, % B 22; 30 min, % B 22, 35 min, % B 25; 40 min, % B 35; 50 min, % B 35; 60 min, % B 45; 65 min, % B 45; 70 min, % B 60. Details of gradient control are given in Hewlett-Packard technical literature. The eluted peptides were collected as indicated by the bars. The amino acid compositions of these peptides are given in Table 3 (supplementary material), and the amino acid sequences of these peptides are summarized in Figure 1.

1. Automated sequence analysis (Table 5, supplementary material) indicated that peptide R1 overlapped peptides CB1 and CB9 and contained residues 75–106. Peptide R2 contained the single histidinyl residue as well as a tyrosyl residue, and automated sequence analysis (Table 5, supplementary material) yielded 32 cycles of degradation with unambiguous assignments, which indicated that peptides CB9 and CB6 were contiguous.

In order to further characterize peptide R2, it was subdigested with trypsin, and the products were isolated by high-performance liquid chromatography (Figure 9, supplementary material). The amino acid compositions of peptides R2-A through R2-F are given in Table 4 (supplementary material), and the automated sequencer determinations of peptides R2-B through R2-F are given in Table 6 (supplementary material). Peptide R2-B gave the sequence of residues 107–115, peptide R2-E had the sequence of residues 116–126, and peptide R2-F corresponded to residues 127–143. The carboxy-terminal residues of R2 were more completely defined by the sequences of peptides R2-A, -C, and -D. Peptide R2-C contained the carboxy-terminal sequence of residues 114–149, including the sequence of peptide CB2. The proof of the connection between peptides R2-F and R2-C is weak because the terminal homoserine was not detected in the sequence analysis of peptide CB6. Peptide R2-D contained residues 144–148, indicating only partial trypsin cleavage of the bond between residues 148 and 149. The recoveries of peptides R2-C and -D together are similar to those for the other peptides, indicating that they do not represent products from different cleavage sites. By use of fluorescence detection of unhydrolyzed material, peptide R2-A was shown to contain asparagine in a yield similar to that of peptide R2-D. Thus, peptides R2-A, -C, and -D account for the carboxy-terminal hexapeptide.

Peptide R3 (Figure 1) had an amino acid composition similar to the amino-terminal 37 residues of bovine brain calmodulin (Figure 2). Therefore, peptide R3 was subdigested with trypsin for further sequence analysis. Three peptide products were isolated by reverse-phase high-performance liquid chromatography, as shown in Figure 10 (supplementary material). The amino acid compositions of peptides R3-A through R3-C are shown in Table 4 (supplementary material) and the automated sequencer determinations of peptides R3-A and R3-B are given in Table 7 (supplementary material). Peptide R3-A contained the single arginyl residue from peptide R3, and upon sequence analysis (Figure 1), peptide R3-A indicated that peptides CB11-A and CB3 were contiguous. The sequence of peptide R3-B (Figure 1) was identical with that of peptide CB11-B, and the amino acid composition of peptide R3-C was similar to that of peptide CB11-C.

The peptide fraction R4 contained a doublet in the chromatographic analysis shown in Figure 3. The amino acid composition of pool R4 (Table 3, supplementary material) is consistent with a mixture of peptides homologous to residues 38–74 and 38–106 of bovine brain calmodulin (Figure 2), in the relative amounts of 70 and 30%, respectively. These data indicated partial cleavage at arginine-74 (Figure 1) and accounted for the relatively low yield (44%) of peptide R1, residues 75–106. The recovery of pool R4 (94%) was calculated from the recovery of proline, on the basis of a ratio of 2 mol of proline/mol of protein. Sequence analysis (Table 8, supplementary material) of the first 37 residues of pool R4 gave a single sequence, which ordered peptides CB3, CB7, and CB1.

Quantitative Activator Activities. In order to determine whether the structural differences between *Dictyostelium* and bovine calmodulins correlated with functional activity, *Dictyostelium* calmodulin was tested for enzyme activator activities. We selected bovine brain cyclic nucleotide phosphodiesterase because most chemically homogeneous calmodulins exhibit indistinguishable activation of this enzyme (Klee & Vanaman, 1982). As shown in Figure 4, no significant difference was detectable in the activation of bovine brain phosphodiesterase by chemically homogeneous calmodulin from bovine brain or *Dictyostelium* under the conditions of the assay. The activation of NAD kinase was tested because phylogenetically distinct calmodulins with structural differences have been shown to differ in their activation of this enzyme (Jarrett et al., 1982). The maximal activity of NAD kinase was 4.6-fold greater in the presence of saturating amounts of *Dictyostelium* calmodulin than in the presence of bovine brain calmodulin, as shown in Figure 5. Similar amounts of *Dictyostelium* or bovine calmodulin were required for half-maximal activation of NAD kinase.

Discussion

We have purified calmodulin from *Dictyostelium* to apparent homogeneity and have characterized the protein in terms of its nearly complete amino acid sequence and quantitative ability to activate cyclic nucleotide phosphodiesterase from bovine brain and NAD kinase from pea. As part of the amino acid sequence analysis, we developed a new protocol for the selective and limited cleavage of calmodulin using an arginine-specific endopeptidase from mouse submaxillary gland. This cleavage results in several large peptides that can be readily separated in high yield by high-performance liquid chromatography (Figure 3) and either directly subjected to automated sequencer analysis or further digested with other proteases. These large peptides, which can be rapidly isolated in high yields, might be useful in studies of the functional domains of calmodulin.

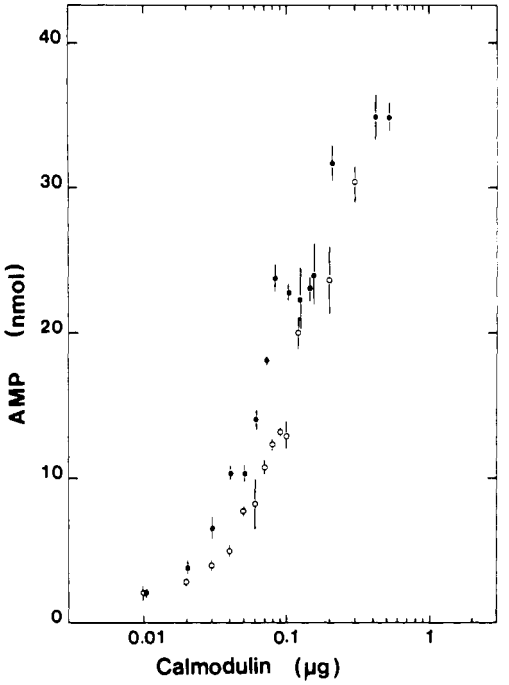

FIGURE 4: Activation of bovine cyclic nucleotide phosphodiesterase by *Dictyostelium* (●) or bovine brain (○) calmodulin. Each assay mixture contained 15 μg of phophodiesterase fraction, 40 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$, 0.4 mM $MnCl_2$, 2 mM cAMP, and various amounts of calmodulin in a final volume of 0.25 mL. Assays were incubated for 6 min at 30 °C, and the substrate and product were separated by high-performance liquid chromatography on a column (0.46 × 25 cm) of anion-exchange resin (Partisil-10 SAX). The amount of AMP was quantitated by its absorbance at 254 nm as previously described (Watterson et al., 1980c). The ordinate shows the nanomoles of AMP produced in each assay mixture, and the abscissa shows the amount of calmodulin added. The points shown are the means of duplicate determinations with the range shown by the bars.

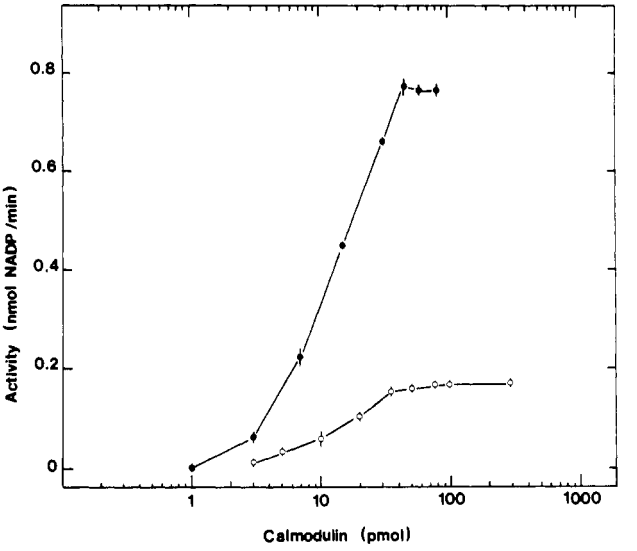

FIGURE 5: Activation of pea seedling NAD kinase by *Dictyostelium* (●) or bovine brain (○) calmodulin. Each assay mixture contained 3 mM ATP, 2 mM NAD, 1 mM $CaCl_2$, 10 mM $MgCl_2$, 50 mM KCl, 50 mM Tris-HCl, pH 8.0, 0.025 mL of enzyme preparation, and various amounts of calmodulin in a final volume of 0.50 mL. The reactions were initiated by the addition of the enzyme preparation and were incubated for 30 min at 37 °C. The reactions were terminated by boiling for 5 min, and the NADP produced was determined according to the method of Apps (1970). The ordinate shows the activity as the nanomoles of NADP produced per minute in each assay mixture, and the abscissa shows the amount of calmodulin added. The points shown are the means of duplicate determinations with the range shown by the bars.

Dictyostelium calmodulin appears to consist of 151 amino acids, of which 146 residues were placed in a linear sequence, as shown in Figure 1. Fourteen amino acid sequence differences between *Dictyostelium* and bovine calmodulin were identified at positions 26, 62, 77, 81, 90, 111, 118, 130, 139, 143, 146, 147, 148, and 149 (Figure 2). All of these differences have been confirmed in separate automated sequencer analyses of different preparations of peptides. Substitutions at positions 26, 81, 90, 130, 139, and 148 conserve the charge or hydrophobic nature of the side chains found at those positions in the bovine brain protein. Asparagine-118 replaces an aspartic acid, neutralizing one negative charge at neutral pH. Glutamine-77 replaces a lysine, but lysine-143 replaces a glutamine, leading to no additional change in the net charge of the protein. These substitutions could affect the conformation or reactivity of the protein in particular domains.

Seven of the amino acid substitutions in *Dictyostelium* calmodulin are novel in that the amino acid residues at these positions are invariant in all calmodulin sequences previously examined (Figure 2). Six of these novel substitutions (positions 62, 77, 81, 111, 139, and 148) are compatible with single nucleotide changes from the codon structure of bovine calmodulin. In particular, the substitutions at positions 77 and 81 alter a highly conserved region that has been postulated to be involved in the interaction of calmodulin or troponin C with troponin I (Perry et al., 1979). Bazari & Clarke (1981) found that *Dictyostelium* calmodulin and vertebrate calmodulin differed in their interactions with troponin I when analyzed by polyacrylamide gel electrophoresis in the presence of urea. These observations suggest that detailed studies of the interactions between chemically homogeneous *Dictyostelium* calmodulin and troponin I may add to our understanding of a protein binding domain of calmodulin.

Dictyostelium calmodulin also contains an additional residue, an asparagine, on the carboxy terminus of the protein. This is the first demonstration of a calmodulin that contains a carboxy-terminal extension. The nucleotide codon for asparagine is a minimum two-nucleotide change from a termination codon. However, both bovine cardiac and rabbit skeletal muscle troponin C have residues in a homologous position (Watterson et al., 1980a), glutamic acid and glutamine, respectively. The amino acid composition of peptide TH-2 suggests the possibility of an amino-terminal extension in *Dictyostelium* calmodulin. If subsequent studies demonstrate an extension, this would also be similar to the amino-terminal extensions found in the troponin C members of the calcium-modulated protein family.

Substitutions at positions 26, 90, 118, 130, 143, 146, and 147 of bovine brain calmodulin have been found in several species examined. Spinach calmodulin contains a single residue of cysteine at position 26 (Watterson et al., 1983), which is compatible with a minimum two-nucleotide change in the codon structure of the bovine protein (threonine-26). However, serine-26 in *Dictyostelium* calmodulin is compatible with a minimum one-nucleotide change from either the bovine (threonine) or the spinach (cysteine) codon. Thus, *Dictyostelium* calmodulin is intermediate in structure between a vertebrate and a higher plant calmodulin at position 26.

Bovine, spinach, *Renilla*, and *Tetrahymena* calmodulins have a trimethyllysine at position 115, while *Chlamydomonas* calmodulin has an unmethylated lysine at this position (Schleicher et al., 1984). Sequence analysis of peptides CB9 and R2 from *Dictyostelium* calmodulin clearly indicated lysine at the homologous position (115), and the lysinyl–leucinyl peptide bond at residues 115 and 116 was cleaved by trypsin

in the subdigestion of peptide R2. This cleavage indicates that methylation of lysine-115 protects the site from trypsin digestion as previously suggested (Van Eldik et al., 1980). The apparent lack of methylation of lysine-115 may be due to the lack of availability of a methylating enzyme in *Dictyostelium*, since Rowe et al. (1983) have found that *Dictyostelium* calmodulin serves as substrate for a mammalian *S*-adenosylmethionine:lysine *N*-methyltransferase. The ability to methylate *Dictyostelium* calmodulin in vitro should make it possible to compare the interaction of methylated and unmethylated forms of the protein with calmodulin-regulated enzymes. As shown here, the lack of methylation of lysine-115 does not appear to affect the ability of *Dictyostelium* calmodulin to activate bovine brain cyclic nucleotide phosphodiesterase in vitro.

Because calmodulin has multiple functions in vitro, the novel structural differences in *Dictyostelium* calmodulin may affect various calmodulin-regulated enzymes differently. As shown in the present study, *Dictyostelium* and bovine calmodulin are indistinguishable in their activation of bovine brain cyclic nucleotide phosphodiesterase. However, *Dictyostelium* calmodulin activates pea NAD kinase to a higher maximal level than does bovine calmodulin. Differences in quantitative activator activities among various calmodulins have been reported for several enzymes. Klumpp et al. (1983) reported that guanylate cyclase activity from *Paramecium* could be activated by vertebrate, higher plant, or protozoan calmodulin but not by *Dictyostelium* calmodulin. Kakiuchi et al. (1981) found that *Tetrahymena* guanylate cyclase could be activated only by calmodulin from *Tetrahymena*. Jarrett et al. (1982) reported that different amounts of peanut or porcine brain calmodulin were required for half-maximal activation of pea NAD kinase. Thus, the limited number of amino acid sequence differences in calmodulins appears to have functional significance.

Further studies of *Dictyostelium* calmodulin and its interaction with receptor proteins may provide information on the precise structural basis of these functional differences. In particular, proteins in *Dictyostelium* should be examined in order to determine whether there are any enzymes that interact preferentially with *Dictyostelium* calmodulin. Quantitative calcium and magnesium ion binding studies are also required to establish how *Dictyostelium* calmodulin interacts with these ions and to compare these interactions with those of other calmodulins. The availability of mutants with conditional defects in motility (Kayman et al., 1982) and in the production of or response to cAMP (Barclay & Henderson, 1977) should facilitate studies of the role of calmodulin in *Dictyostelium* cell function.

Comparison of the *Dictyostelium* calmodulin sequence with all calmodulins for which amino acid sequence data are available reveals that the region of greatest extended sequence identity extends from residues 27 to 58 (Figure 2). This highly conserved region includes half of the proposed calcium binding structures of domains 1 and 2 and the interdomain region between domains 1 and 2. This observation suggests that this region of extended sequence identity may be particularly important to calmodulin activities that serve a fundamental role in cell function.

Models of the evolution of calmodulin propose that this and other calcium-modulated proteins may have evolved by duplication of a one-or two-domain precursor (Vanaman et al., 1977; Erickson et al., 1980). The internal homologies among the domains of *Dictyostelium* calmodulin support these proposals. As in other calmodulins, domain 1 has the highest homology with domain 3, and domain 2 has the highest homology with domain 4. In addition, *Dictyostelium* calmodulin has an extension at the carboxy-terminus end, as does troponin C from two mammalian sources. *Dictyostelium* calmodulin may also have an extension at the amino terminus. Thus, in these respects, *Dictyostelium* calmodulin may represent a structure that is closer to a common precursor than any calmodulin or troponin C molecule characterized. Goodman (1980) has suggested that the rate of evolution of calmodulin was discontinuous, involving rapid evolution concomitant with the emergence of the basal Eukaryota and very slow evolution during the past billion years. Our studies of *Dictyostelium* calmodulin are consistent with this hypothesis and suggest that the slow evolution of calmodulin began prior to the emergence of the Order Acrasiales.

Acknowledgments

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Supplementary Material Available

Tables 3 and 4 giving the amino acid compositions of peptides from the submaxillary gland protease digest and trypsin subdigestions, Tables 5–11 giving the automated sequencer determinations of the peptides, and Figures 6–10 giving the chromatographic results of the cyanogen bromide digest and the trypsin and thermolysin subdigestions (19 pages). Ordering information is given on any current masthead page.

Registry No. NAD kinase, 9032-66-0; cyclic nucleotide phosphodiesterase, 9040-59-9.

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