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Spinach Calmodulin: Isolation, Characterization, and Comparison with Vertebrate Calmodulins†

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ABSTRACT: Calmodulin is the name proposed for a multifunctional, calcium binding protein whose presence has been detected in a number of eukaryotic cells. In the studies summarized here, calmodulin has been isolated from spinach leaves (*Spinacea oleracea*), characterized, and compared to vertebrate calmodulins. Quantitative recovery data for a rapid-isolation protocol demonstrate that calmodulin is a major constituent of spinach leaves. Spinach calmodulin is indistinguishable from vertebrate calmodulins in phosphodiesterase activator activity using vertebrate brain phosphodiesterase and in quantitative immunoreactivity using antiserum made against vertebrate calmodulin. However, spinach calmodulin is readily distinguished from vertebrate and invertebrate calmodulins in electrophoretic mobility and in amino acid composition. Spinach calmodulin, like vertebrate calmodulins, lacks tryptophan and contains 1 mol each of N^{ϵ}-trimethyllysine and histidine per 17 000 g of protein. In contrast to vertebrate calmodulins, spinach calmodulin has only one tyrosinyl residue and has a threonine/serine ratio of 1.3. While amino acid compositions indicate differences between spinach and vertebrate calmodulins, isolation and characterization of tryptic peptides containing the single histidinyl and N^{ϵ}-trimethyllysyl residues and both prolinyl residues indicate that these regions in spinach calmodulin are similar to the corresponding regions in vertebrate calmodulin. These studies more fully define the general and specific characteristics of calmodulins and indicate that calmodulin structure is not as highly conserved among all eukaryotes as it is among vertebrates and invertebrates.

Calcium-modulated proteins are a subgroup, or family, of calcium-binding proteins. It has been proposed [for a review see Kretsinger (1980)] that the biochemical activity, as well as the tertiary structure, of these proteins is modulated by calcium. Members of this group include parvalbumins, troponin C's, and calmodulins. Calmodulin is a multifunctional calcium modulated protein that has been isolated from a variety of eukaryotes [for a review, see Kretsinger (1980); Klee et al., 1980].

Phylogenetic studies of calmodulins and other calcium-modulated proteins have provided information on the common as well as unique structural features of these biological signal transducers. Alignment of the amino acid sequence of bovine brain calmodulin and striated muscle troponin C's clearly demonstrates the structural relationships within this class of calcium-modulated proteins (Watterson et al., 1980a). Comparison of the amino acid sequence repeats found in the primary structures of calmodulin and troponin C suggests that these proteins have arisen by duplication of a two-domain precursor (Watterson et al., 1980a). Using algorithms for the comparison of calcium-modulated proteins, Barker et al. (1978) and Erickson et al. (1980) have suggested that calmodulin is closely related to the original four-domain precursor of calmodulin, troponin C, and the myosin light chains. Therefore, structural and functional comparisons of calmodulins from closely related as well as distant species might provide insight into the evolution of this class of biologically important macromolecules.

Reports of amino acid sequences for calmodulins from a limited number of sources (Watterson et al., 1980a; Dedman et al., 1978; Grand & Perry, 1978) as well as detailed comparative characterization studies (Watterson et al., 1980b) indicate that the calmodulin molecule has been highly conserved during vertebrate evolution. Calmodulin or calmodulin-like proteins have been isolated from only a few invertebrate

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and plant species. As with many of the early reports on vertebrate calmodulins (for a discussion see Watterson et al., 1980b), there appear to be a number of major differences in the reported chemical and physical properties of calmodulins from these "lower" species. Most interesting among the differences between invertebrate and vertebrate calmodulins is the report (Waisman et al., 1978) that calmodulin from the earthworm (*Lumbricus terrestris*) binds only 2 mol of calcium/mol of protein, contains cysteine, and has a tryptic peptide map that shows several differences from that of bovine brain calmodulin. Calmodulin from the electroplax of the electric eel *Electrophorus electricus* has been reported (Childers & Siegel, 1975) to be a phosphoprotein of 14 000 molecular weight, yet to have an amino acid composition and peptide map indistinguishable from those of vertebrate brain calmodulin. Calmodulin from the marine invertebrate *Renilla reniformis* has been shown (Jones et al., 1979) to be very similar to bovine brain calmodulin but reported to have several amino acid sequence differences (Sharief & Vanaman, 1979). Calmodulin from the sea urchin egg (*Arabacia punctulata*) has been shown (Head et al., 1979) to resemble the bovine brain protein but to have a different peptide map. Plant calmodulins have been studied (Anderson & Cormier, 1978) in peas (*Pisum sativum*) as a subunit of NAD kinase and have been isolated from a limited number of other species (Wang & Waisman, 1979). However, little data are available in the literature (Charbonneau & Cormier, 1979; Wang & Waisman, 1979) concerning the chemical characterization of plant calmodulins.

Calcium is clearly important in the regulation of plant function (Anderson & Cormier, 1978; Hale & Roux, 1980). However, the various roles of calmodulin and other calcium-modulated proteins in plant cell function are not known. Detailed studies of calcium-modulated proteins from plants, especially a ubiquitous, multifunctional protein such as calmodulin, would contribute to our knowledge of metabolic regulation by calcium. Spinach (*Spinacea oleracea*) has been studied extensively with respect to protein evolution (Boulter et al., 1972) and physiological mechanisms (Jensen & Bahr, 1977). Therefore, as part of a detailed study of the structure, function, and evolution of calcium-modulated proteins, we have isolated and characterized calmodulin from spinach leaves and directly compared it to the well-characterized vertebrate protein. The data reported here demonstrate that plant calmodulin differs from the highly conserved vertebrate calmodulins and suggest that plant calmodulins might be excellent sources for future structure–function studies of calcium-modulated proteins. A preliminary report of some of the conclusions of this work has been presented (Van Eldik et al., 1980).

Materials and Methods

Calmodulin was isolated exactly as previously described (Van Eldik & Watterson, 1979) and by a more rapid procedure, described below, which is a modification of previously described procedures (Van Eldik & Watterson, 1979; Jamieson & Vanaman, 1979). Spinach leaves were rinsed, drained, and diced. The diced material (usually 1–5 kg wet weight) was homogenized at 4 °C in 0.5 volume (500 mL/kg, wet weight) of buffer H (0.05 M Tris-HCl, 0.001 M EGTA,[1] and 0.001 M β-mercaptoethanol, pH 8.0). Homogenization was done at low speed in a Waring blender until a thick puree with minimal foam was formed. An additional 0.5 volume of buffer H was added and the suspension stirred slowly at 4 °C for 5–10 min. The suspension was filtered through cheesecloth and filtrate saved. The green but relatively clear filtrate was adjusted to 55% saturation by the slow addition of granular, solid ammonium sulfate (351 g/L). The suspension was stirred at 4 °C for 0.5–1.0 h, and insoluble material was removed by centrifugation at 10000*g* for 1 h. The supernatant was adjusted to pH 4.1 with 50% (v/v) sulfuric acid and stirred for 1–2 h at 4 °C. The resultant precipitate was collected by centrifugation at 10000*g* for 1.5 h. The pH 4.1 supernatant was discarded, and the pellet was redissolved in buffer B (0.01 M Tris–HCl, 0.001 M EGTA, 0.001 M β-mercaptoethanol, and 0.20 M NaCl, pH 8.0), the pH was adjusted to 7.4, and the solution was dialyzed overnight against a large volume of buffer B. The conductivity and pH of the dialysate were determined, and then particulate matter was removed by centrifugation at 40000*g* for 1 h.

The dialyzed and clarified sample was subjected to chromatography on DEAE–Sephadex A-50 as previously described (Watterson et al., 1976). The fractions containing calmodulin from the ion-exchange chromatography step (conductivity at 25 °C was approximately 19 $m\Omega^{-1}$) were pooled, dialyzed against deionized water, and lyophilized to dryness. The resultant powder was redissolved in buffer F (0.01 M Tris-HCl, 0.001 M $MgCl_2$, 0.001 M β-mercaptoethanol, and 0.002 M $CaCl_2$, pH 8.0), and the clear solution was subjected to chromatography on phenothiazine–Sepharose conjugates as described below. Calmodulin was monitored during purification by electrophoresis on discontinuous gels in the absence of detergent as previously described (Van Eldik et al., 1980) and by phosphodiesterase activator activity (Watterson et al., 1980c). Multiple dilutions of each fraction were boiled and centrifuged in a Microfuge, and the ability of these supernatants to stimulate phosphodiesterase was compared to a standard curve of bovine brain calmodulin. Only those portions of the activation curve were used where activity was proportional to protein concentration and the slope parallel to the calmodulin standard curve.

Phenothiazine–Sepharose conjugates were prepared and used essentially as previously described (Van Eldik et al., 1980). Samples were applied to a column (2.5 × 7.5 cm) of phenothiazine–Sepharose equilibrated with buffer F. The column was subsequently washed with buffer F and buffer F containing 0.2 M NaCl until absorbance at 280 nm was at base line. Calmodulin was eluted with buffer E (0.01 M Tris-HCl, 0.001 M $MgCl_2$, 0.001 M β-mercaptoethanol, and 0.002 M EGTA, pH 8.0) containing 0.2 M NaCl, and the column was subsequently washed with buffer E containing 8.0 M urea.

Trypsin digestions were performed in the presence of EGTA as previously described (Watterson et al., 1976). The elution patterns of peptides were determined on a Whatman Partisil M9 ODS-2 column (9.4 × 250 mm) by using a modified (Watterson et al., 1980c) Hewlett-Packard 1084B liquid chromatography system. Solvent A was 0.1% (v/v) hydrochloric acid (J. T. Baker, Ultrex), and solvent B was acetonitrile (UV grade). The microprocessor controlled elution gradient consisted of the following time program: 0–5 min, % B = 5; 5.5 min, % B = 10; 10 min, % B = 10; 20 min, % B = 25; 30 min, % B = 25; 40 min, % B = 35; 50 min, % B = 35; 60 min, % B = 45; 70 min, % B = 45; 71 min, % B = 5; 80 min, run was stopped.

Samples for amino acid analysis were hydrolyzed, and composition analyses were done as previously described (Van Eldik & Watterson, 1979). Tryptophan was determined after

[1] Abbreviations used: EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; N^{ϵ}-trimethyllysine, $N^{\epsilon},N^{\epsilon},N^{\epsilon}$-trimethyllysine.

Table I: Purification of Spinach Calmodulin[a]

purification step	total vol (mL)	protein concn[b] (mg/mL)	total protein (mg)	total calmodulin[c] (mg)	yield[d] (%)	purification[d] (x-fold)
(1) homogenization	2800	1.51	4228	26	100	*e*
(2) 55% $(NH_4)_2SO_4$, pH 7.0	3465	0.31	1074	16	62	2
(3) 55% $(NH_4)_2SO_4$, pH 4.0	97	1.40	136	15	58	18
(4) DEAE-Sephadex A-50	96	0.13	12	8	31	115
(5) phenothiazine-Sepharose	*f*	*f*	8	8	31	164

[a] Data given are from a representative purification starting with ~1 kg (wet weight) of leaves. [b] Determined by the method of Lowry et al. (1951). [c] Determined by phosphodiesterase activator activity as described in the text. [d] For percentage of yield, the amount of calmodulin present at step 1 was assumed to be 100%. Purification was calculated as total calmodulin/total protein at each step divided by total calmodulin/total protein at step 1. [e] Not significant. [f] Lyophilized solid.

hydrolysis in methanesulfonic acid (Simpson et al., 1976). N^{ϵ}-Trimethyllysine was detected and quantitated as described (Van Eldik et al., 1980). Peptide cleavage at cysteine after treatment with 2-nitro-5-thiocyanobenzoate was done according to the protocol of Stark (1977). Quantitative NH_2-terminal determinations were done as previously reported (Van Eldik & Watterson, 1979) by using automated Edman degradation with identification of the resultant phenylthiohydantoin derivatives by reverse-phase chromatography.

Antibody against vertebrate calmodulin was prepared, and radioimmunoassays were done as reported elsewhere (Van Eldik & Watterson, 1980). Acrylamide and *N,N'*-methylenebisacrylamide were obtained from Atomergic Chemetals Corp. Molecular weight standards for polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate were purchased from Sigma and Boehringer Mannheim. Distilled, deionized water was from a Darco Water System (Durham, NC). N^{ϵ}-Trimethyl-L-lysine bis(*p*-hydroxyazobenzene-*p*-sulfonate) was purchased from Calbiochem and converted to the acetate form by passage through a column of Bio-Rad AG-1-X8 resin (200–400 mesh, acetate form). Spinach leaves were obtained fresh from a local vendor. All other chemicals were of the highest purity reagent grade commercially available and used without further purification.

Results

Calmodulin was purified from spinach leaves by using standard fractionation procedures previously described (Van Eldik & Watterson, 1979) and affinity chromatography procedures as summarized under Materials and Methods. As shown in Table I, calmodulin represents ~0.6% of the soluble protein present in the initial spinach homogenate filtrate. While the yield for the isolation shown in Table I is 31%, yields have ranged from 20% to 31%. The material that does not bind to the phenothiazine–Sepharose is routinely examined for the presence of calmodulin to ensure that the majority of the calmodulin has been recovered. The fold purification at the DEAE–Sephadex chromatography and ammonium sulfate steps is similar to that previously reported (Watterson et al., 1976) in the purification of bovine brain calmodulin using standard procedures. However, the substitution of three column chromatography steps and one centrifugation step with a single affinity chromatography fractionation results in a greater overall yield. Purification time is also significantly reduced.

Purified calmodulin preparations were analyzed as summarized under Materials and Methods by polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate, in the presence and absence of calcium, and in the presence and absence of EGTA. As shown in Figure 1 for analysis in the presence of EGTA and sodium dodecyl sulfate, the calmodulin preparations consisted of a single Coomassie blue staining band. With protein loadings in the 50–200-μg range, higher molecular weight contaminants were occasionally detected in some preparations. When these contaminants were present, they were readily removed by a second phenothiazine–Sepharose or DEAE–Sephadex chromatography step. Lower molecular weight contaminants were detected when aged spinach was used as the starting material. These contaminants were usually avoided by starting with fresh spinach leaves. When necessary, these lower molecular weight contaminants were removed by chromatography on DEAE–Sephadex.

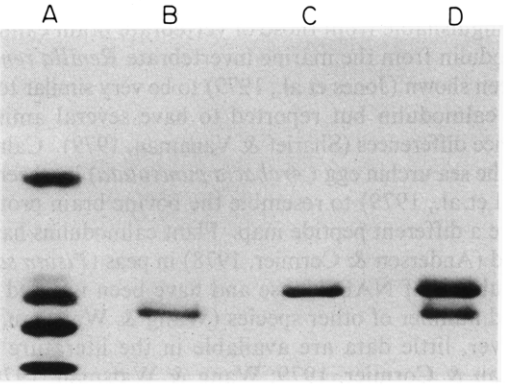

FIGURE 1: Electrophoretic analysis of spinach calmodulin. Electrophoresis was done in the presence of sodium dodecyl sulfate on a 12.5% (w/v) acrylamide gel containing 1 mM EGTA. All samples and gel buffer also contained 1 mM EGTA. Samples were incubated at 100 °C for 3 min before analysis. Lane A contained 5 μg of each molecular weight standard. From top to bottom, the standards are chymotrypsinogen, soybean trypsin inhibitor, myoglobin, and lysozyme. Lane B is 5 μg of spinach calmodulin, lane C is 5 μg of bovine brain calmodulin, and lane D is a mixture of 5 μg each of bovine brain calmodulin and spinach calmodulin.

When spinach leaf calmodulin and bovine brain calmodulin were analyzed in the presence of calcium and sodium dodecyl sulfate, the two proteins comigrated and had an anomalously low estimated molecular weight of 13 000–15 000. When spinach leaf and bovine calmodulins were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and EGTA, the two calmodulins did not comigrate (Figure 1). The estimated molecular weight of bovine brain calmodulin relative to protein standards under these conditions was in the range of 18 000–20 000. However, the calculated molecular mass of bovine brain calmodulin is 16 680 g/mol (Watterson et al., 1980a). The estimated molecular weight of spinach leaf calmodulin under the electrophoretic conditions of Figure 1 was 17 000–19 000. When bovine brain and spinach leaf calmodulins were mixed and subjected to electrophoresis (Figure 1), a difference in molecular weight of ~1000 was obtained. Similarly, rabbit skeletal muscle troponin C has a molecular weight of 17 965 (Collins et al., 1977), and, in this same gel system (Figure 1), troponin C and

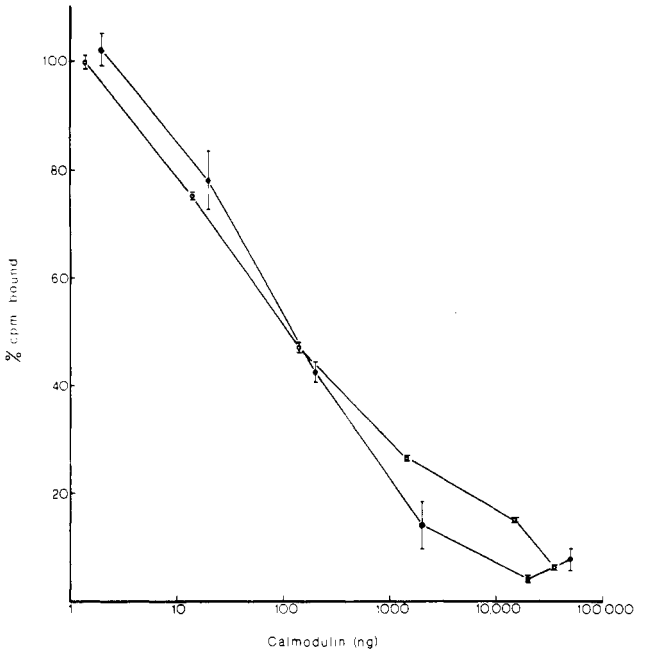

FIGURE 2: Comparison of spinach calmodulin and bovine brain calmodulin by competition radioimmunoassay. Various concentrations of spinach calmodulin (○) and bovine brain calmodulin (●) were mixed with anti-calmodulin serum and incubated overnight at 4 °C. A fixed amount of ^{125}I-labeled bovine brain calmodulin (1 ng; 50 000 cpm) was added, and the reaction was incubated overnight at 4 °C and then processed as described under Materials and Methods. The degree of competition is expressed as a percent of the cpm bound in the absence of competing antigen.

calmodulin migrated with an apparent molecular weight difference of ~1500 (data not shown). The determination of the exact molecular weight of spinach calmodulin must await the elucidation of its amino acid sequence. Until the exact molecular weight of spinach calmodulin has been determined, two molecular weights will be used in the calculations of amino acid compositions: (1) an estimated molecular weight (15 680) based on difference comparison in gel electrophoresis to other calmodulins and troponin C and (2) the known molecular weight (16 680) of bovine brain calmodulin.

The ability of spinach leaf calmodulin to stimulate calmodulin-deficient bovine brain phosphodiesterase was compared to that of the homologous bovine brain calmodulin. Stock solutions of bovine brain and spinach leaf calmodulins were quantitated by amino acid analysis. Dilutions of each stock solution were tested in triplicate for their ability to quantitatively stimulate 15 μg of bovine brain phosphodiesterase as described under Materials and Methods. The final concentration of substrate, cyclic AMP, was 2 mM in a reaction volume of 250 μL. Substrate and product in the reaction mixture were quantitated by high-performance liquid chromatography. Half-maximal stimulation of phosphodiesterase activity (9.5 nmol produced in 250 μL per 6 min) was obtained with 60 ng of both spinach leaf calmodulin and bovine brain calmodulin. The activation curves for spinach leaf calmodulin and bovine brain calmodulin were superimposable.

As shown in Figure 2, spinach leaf calmodulin was also indistinguishable from vertebrate calmodulins in immunoreactivity. The antisera used in the competition radioimmunoassay studies were prepared against vertebrate calmodulin, and the iodinated standard was the homologous vertebrate protein. The antiserum showed no reactivity against parvalbumin or S100b. The competition curve for the purified spinach calmodulin was not significantly different from that of the vertebrate calmodulin (Figure 2). Thus the antigenic determinants in the spinach and vertebrate calmodulin molecules appear to be conserved as well as the domains required for phosphodiesterase activation.

Table II: Amino Acid Compositions of Calmodulin[a]

amino acid	spinach leaf[b] residues/ 15 680 g	spinach leaf[b] residues/ 16 680 g	bovine brain[c] residues/ molecule
aspartic acid	23.5	25.0	23
threonine	7.3	7.8	12
serine	5.6	6.0	4
glutamic acid	29.7	31.7	27
proline	1.8	1.9	2
glycine	10.3	11.0	11
alanine	10.5	11.1	11
half-cystine	N.D.[d]	N.D.	0
valine	4.9	5.2	7
methionine	7.0	7.5	9
isoleucine	5.0	5.3	8
leucine	10.5	11.2	9
tyrosine	1.0	1.0	2
phenylalanine	7.5	8.0	8
histidine	0.9	1.0	1
trimethyllysine	1.0	1.0	1
lysine	7.7	8.2	7
tryptophan	0	0	0
arginine	4.3	4.5	6

[a] Determined as described in the text. [b] Calculated as described in the text from composition analysis by using the molecular weight calculated from the sequence of bovine brain calmodulin (16 680) and the estimated molecular weight of spinach calmodulin from polyacrylamide gel analysis (15 680). [c] Composition calculated from amino acid sequence (Watterson et al., 1980a). [d] Not determined.

The amino acid composition of spinach leaf calmodulin is shown in Table II along with the composition by sequence of bovine brain calmodulin. The spinach leaf calmodulin composition values have been calculated by using molecular weights of 15 680 and 16 680 (see above). Several of the characteristic features of the amino acid compositions of vertebrate calmodulin are found in spinach leaf calmodulin. These include a high aspartic acid and glutamic acid content, the absence of tryptophan, and the presence of a single residue each of histidine and N^{ϵ}-trimethyllysine. There are three notable differences between the bovine brain and the spinach leaf calmodulin compositions. First, there is one tyrosinyl residue in spinach calmodulin and two tyrosines in bovine brain calmodulin. Second, there are six arginyl residues in bovine brain calmodulin but only five in the spinach protein. Third, spinach calmodulin has a threonine/serine ratio of 1.3 while that found in bovine brain calmodulin is 3.0.

In most calmodulin preparations, a variable amount of a UV-absorbing material was found in the final lyophilized solid. This material, when present, was removed by passage over a small column of Sephadex G-25 (25 × 3.5 cm) equilibrated with 0.10 M ammonium bicarbonate or by reverse-phase chromatography as described under Materials and Methods. Vertebrate calmodulins purified by the same protocol also contained this UV-absorbing material. When spinach and vertebrate calmodulins that contained this unidentified material were oxidized with performic acid and then analyzed for the presence of cysteic acid, approximately 1 mol of material like cysteic acid was detected per 17 000 g of protein. When the calmodulin preparations were treated with iodoacetate and then analyzed for the presence of carboxymethylcysteine, a compound that coeluted with carboxymethylcysteine during amino acid analysis was detected. After gel filtration or reverse-phase chromatography, the amount

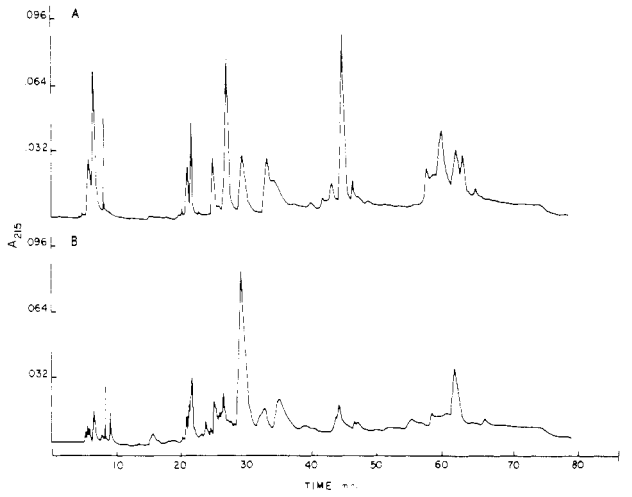

FIGURE 3: Column peptide maps of spinach and vertebrate calmodulins. Purified spinach calmodulin and rabbit brain calmodulin were digested with trypsin as described under Materials and Methods. The traces shown are (A) digest of rabbit brain calmodulin and (B) digest of spinach leaf calmodulin. Each digest was lyophilized and then redissolved in distilled, deionized water. A 20-μL aliquot of each digest was injected ($\sim$4 nmol based on amino acid analysis) and fractionated on a reverse phase column (Whatman ODS-2, 9.4 × 250 mm) by using the elution gradient given under Materials and Methods.

of this unidentified material in vertebrate calmodulin preparations was decreased. Finally, when vertebrate and spinach calmodulins were treated with cysteine-specific peptide cleavage reagent 2-nitro-5-thiocyanobenzoate (Stark, 1977) and then analyzed by polyacrylamide gel electrophoresis, vertebrate calmodulin was not altered in its electrophoretic mobility whereas spinach calmodulin was quantitatively converted to faster migrating bands. Because of these ambiguities in data obtained with relatively homogeneous preparations of calmodulin, the cysteine content of spinach calmodulin can only be given as not determined (Table II). More detailed studies including amino acid sequence analysis are required in order to unequivocally determine whether cysteine is present in spinach calmodulin.

Because comparative tryptic peptide maps have been used to demonstrate sequence relatedness of calmodulins (Stevens et al., 1976; Van Eldik & Watterson, 1979; Watterson et al., 1980b), the elution patterns of peptides from spinach leaf calmodulin and vertebrate calmodulin were compared. Spinach calmodulin and rabbit brain calmodulin were digested with trypsin in the presence of EGTA as described under Materials and Methods. The amount of protein injected onto the column was determined by amino acid analysis of an aliquot of the digest taken immediately before injection. The elution profiles of the two digests are shown in Figure 3. A control digest containing all reagents except calmodulin was also analyzed. Under the conditions of analysis, the control digest gave essentially a base-line profile. The use of 215-nm monitoring for peptide purification and fractionation allows maximal sensitivity in a portion of the ultraviolet spectrum in which commonly used solvents are relatively transparent. In general, absorption of ultraviolet light at 215 nm by a protein or peptide is due to multiple factors, e.g., aromatic residues, peptide bonds, some types of hydrogen bonds, and other interactions dependent on conformation and helix content (White et al., 1978). The peak heights in Figure 3 can only be interpreted qualitatively because of such multiple contributions. The presence of a peak at a given time, however, is quite reproducible. As in other column mapping procedures (Van Eldik & Watterson, 1979) and in two-dimensional maps on paper (Watterson et al., 1980b), all vertebrate calmodulin digests examined were indistinguishable in this system.

In order to assure that all digest products were recovered, effluent fractions were collected throughout the gradient, and amino acid analysis was done on each fraction. On the basis of the amount of digest injected, recovery of each amino acid ranged from 60% to 80%. The elution profile for spinach calmodulin is similar to the elution profile for the vertebrate calmodulin. Consistent with its lower arginyl plus lysyl content by amino acid analysis (Table II), the spinach calmodulin digest appears to have fewer peptides. In both digests, free lysine eluted near the void volume and before the first peak shown in Figure 3. This was the only digest product for which no absorbance peak was detected. Under these analysis conditions, 4 nmol of lysine does not give a detectable photometer response.

In studies of the amino acid sequence of bovine brain calmodulin (Watterson et al., 1980a), three tryptic peptides (peptides T-7, T-1A, and T-5A) were readily identifiable in terms of their amino acid compositions. Peptide T-7 was free lysine and was the smallest tryptic peptide. Peptide T-1A was the largest tryptic peptide isolated and included the two prolinyl residues of calmodulin as well as most of domain 2 of calmodulin. Peptide T-5A included the single residues of histidine and N^{ϵ}-trimethyllysine found in calmodulin. Finally, the regions of bovine brain calmodulin that are included in peptides T-7, T-1A, and T-5A (residues 38–75 and 107–126) have been implicated in calmodulin functions [see Watterson et al. (1980a) for a discussion]. Therefore, the analogous peptides from spinach calmodulin were isolated and characterized. For comparative and experimental control purposes, the same peptides were isolated from the digest of rabbit brain calmodulin whose elution profile is shown in Figure 3A. The compositions of the peptides from rabbit brain calmodulin were indistinguishable from the corresponding peptides from bovine brain calmodulin.

As noted above, free lysine was isolated from the trypsin digest of spinach calmodulin. Free lysine (peptide T-7) was generated from bovine brain calmodulin by cleavage at an arginyllysyl bond at residues 74 and 75 (Watterson et al., 1980a). Amino acid analysis of the major spinach peptide eluting at 62 min (Figure 3B) gave the following (molar ratios): Asp, 8.1; Thr, 2.0; Ser, 1.3; Glu, 7.2; Pro, 2.2; Gly, 3.4; Ala, 2.9; Val, 1.0; Met, 1.9; Ile, 1.8; Leu, 4.2; Phe, 2.0; Arg, 1.0. The amino acid composition of this spinach peptide is extremely similar to that of peptide T-1A (residues 38–74) in the bovine brain sequence (Watterson et al., 1980a). This spinach tryptic peptide had an NH_2-terminal serinyl residue. The only tryptic peptides from bovine brain calmodulin that had an NH_2-terminal serinyl residue were peptides T-1A (residues 38–74) and T-1A′ (residues 38–71) (Watterson et al., 1980a).

The peptide fraction eluting as a skewed peak at 35 min (Figure 3B) contained the single histidinyl and trimethyllysyl residues found in spinach calmodulin. The amino acid composition of this spinach peptide was the following (molar ratios): Asp, 3.0; Thr, 2.3; Glu, 4.0; Gly, 1.3; Val, 1.9; Met, 2.0; Ile, 0.8; Leu, 2.4; His, 1.0; trimethyllysine, 0.8; Arg, 1.0. This composition is indistinguishable from the analogous peptide, T-5A (residues 107–126), from bovine brain calmodulin (Watterson et al., 1980a). Further, the spinach calmodulin peptide had an NH_2-terminal histidinyl residue similar to peptide T-5A from bovine brain calmodulin. In summary, the amino acid compositions, NH_2 termini, and relative elution positions of these spinach peptides indicate that the amino acid

sequences of these spinach calmodulin peptides are very similar to the analogous peptides from bovine brain calmodulin.

Discussion

The studies summarized in this report directly demonstrate that calmodulin is a major constituent of spinach leaves. Spinach leaf calmodulin has been shown to resemble vertebrate calmodulins in phosphodiesterase activation activity, in immunoreactivity, in general physicochemical properties, and in ability to bind to phenothiazine–Sepharose conjugates in a calcium-dependent manner. However, spinach calmodulin differs from vertebrate calmodulins in amino acid composition, in electrophoretic mobility, and in tryptic peptide maps. Burgess et al. (1980) reported that a number of conditions, including protein-bound calcium, can affect the electrophoretic mobility of calmodulins in the presence of sodium dodecyl sulfate. It is not known from the data reported here whether spinach calmodulin actually differs from vertebrate and invertebrate calmodulins in the length of the polypeptide chain or whether it has different calcium binding properties. Amino acid sequence analysis and detailed comparative calcium binding studies, both of which are in progress, will be required in order to unequivocally answer these questions.

Amino acid sequences have been reported for only four calmodulins: bovine brain calmodulin (Watterson et al., 1980a), rat testis calmodulin (Dedman et al., 1978), bovine uterus calmodulin (Grand & Perry, 1978), and *Renilla reniformis* calmodulin (Sharief & Vanaman, 1979). The amino acid sequence of calmodulin from the invertebrate *Renilla reinformis* differs from that of the bovine brain protein in only seven positions, three of which are differences in amidation state (Sharief & Vanaman, 1979). The other four differences can be detected by peptide mapping and by amino acid compositional analysis (Watterson et al., 1980a). Similarly, *Tetrahymena* calmodulin can be distinguished from vertebrate and invertebrate calmodulins by compositional analysis and by peptide mapping (Jamieson et al., 1979). Interestingly, the spinach calmodulin composition reported here is also different from that reported for the *Renilla* (Jones et al., 1979; Jamieson et al., 1979) and *Tetrahymena* proteins (Jamieson et al., 1979). Amino acid sequence analysis of spinach calmodulin will be required in order to unequivocally prove any apparent sequence differences, but the data reported here clearly demonstrate that potentially important structural dissimilarities may exist between vertebrate and plant calmodulins.

During the course of our studies on calmodulin from various plant species, Anderson & Cormier (1978) reported that the calcium-binding subunit of NAD kinase was indistinguishable from vertebrate calmodulin, and Charbonneau & Cormier (1979) isolated calmodulin from other plant species. More recently, Wang & Waisman (1979) have reported on the characterization of plant calmodulins. The ability of purified spinach calmodulin to activate quantitatively bovine brain phosphodiesterase agrees with these previous reports on calmodulin from other plant species. However, the observations that plant and vertebrate calmodulins do not comigrate during gel electrophoresis in the presence of EGTA and sodium dodecyl sulfate and that spinach calmodulin has an amino acid composition different from vertebrate calmodulins appear to be in disagreement with these previous reports (Anderson & Cormier, 1978; Charbonneau & Cormier, 1979; Waisman & Wang, 1979). The basis for these apparent differences is not known but could be due to differences in the methods utilized.

The quantitative immunological cross-reactivity of spinach and vertebrate calmodulins agrees with previous reports (Chafouleas et al., 1979) on the immunological cross-reactivities of calmodulins and calmodulin-like proteins from closely related and distant species. The antisera were prepared in each case by different protocols. However, the demonstration in this report that spinach and vertebrate calmodulins have different amino acid compositions and peptide maps demonstrates that identical reactivity in competition radioimmunoassay is not a criterion for structural identity.

In conclusion, these studies further demonstrate the highly conserved nature of calmodulin. However, compositional and electrophoretic differences between spinach calmodulin and other eukaryotic calmodulins suggest that plant calmodulins may be excellent sources for studies on the structure, function, and evolution of calcium-modulated proteins. The various roles of calmodulin in plant function are not known, but the characterization of spinach calmodulin reported here provides a firm basis for biological studies. The presence of calmodulin as a subunit of NAD kinase in plants (Anderson & Cormier, 1978) and the lack of a plant phosphodiesterase analogous to the brain enzyme (D. M. Watterson, unpublished results) suggest a more likely role in the regulation of phosphorylation and transport than in the regulation of cyclic nucleotide metabolism.

Added in Proof

We have isolated tryptic and cyanogen bromide cleavage peptides with amino acid compositions that apparently encompass the entire spinach calmodulin molecule. The amino acid sequences of all of these peptides have not been determined. However, it is clear that the apparent molecular weight difference of 1000 between vertebrate and spinach calmodulin in polyacrylamide gels cannot be explained by a difference in polypeptide chain length of 10 amino acid residues. It is still possible that a deletion or substitution in the spinach calmodulin sequence of one or two amino acid residues that are crucial for detergent or metal binding could account for the gel mobility difference.

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Binding of Regulatory Nucleotides to Aspartate Transcarbamylase: Nuclear Magnetic Resonance Studies of Selectively Enriched Carbon-13 Regulatory Subunit†

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ABSTRACT: Specifically enriched [γ-^{13}C]phenylalanine, -tyrosine, and -histidine have been biosynthetically incorporated into aspartate transcarbamylase from *Escherichia coli*. These nonperturbing NMR probes have been used to characterize the interaction of the regulatory sites on the enzyme with nucleotide effectors. The C_γ carbons of the three tyrosines and four histidines per regulatory chain give narrow, well-resolved resonances, and the signals from the five phenylalanines per chain are partially resolved in the presence of bound inhibitor. Spectral changes in regulatory subunit were monitored as a function of concentration of the inhibitor, CTP, and the activator, ATP. Three histidine residues responded to ATP and CTP in an identical manner while two phenylalanine residues were sensitive to CTP but not ATP binding. The tyrosine resonances were not perturbed by effectors. The chemical shift response of the single observable histidine resonance to bound nucleotides in the reconstituted enzyme was identical with that observed for isolated regulatory subunit. This histidine spectrum was undisturbed by the *T* to *R* conformational transition of the enzyme. The results suggest that the regulatory subunit experiences minimal rearrangement of tertiary structure on binding effectors and that at least one phenylalanine and one histidine residue are present in the region of the CTP binding site.

Aspartate transcarbamylase (ATCase)[1] (EC 2.1.3.2) from *Escherichia coli* catalyzes the formation of carbamyl-L-aspartate from L-aspartate and carbamyl phosphate, the first committed step in the biosynthesis of pyrimidines (Gerhart, 1970; Schachman, 1972; Jacobsen & Stark, 1973). The enzyme serves as a major control point in this metabolic pathway and is subject to inhibition by CTP and activation by ATP. The 310 000-dalton molecule contains two distinct types of subunit, one type for catalysis and the other for regulation. The native hexameric enzyme is composed of two trimeric catalytic subunits of 100 000 daltons each and three dimeric regulatory subunits of 34 000 daltons. Electron microscopy (Richards & Williams, 1972) and X-ray diffraction studies (Monaco et al., 1978) indicate that the catalytic trimers are not in direct contact but instead are linked via the regulatory dimers. The subunits are separable, and each retains its individual function in the isolated state (Gerhart & Schachman, 1965).

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[1] Abbreviations used: NMR, nuclear magnetic resonance; ATCase, aspartate transcarbamylase; PALA, *N*-(phosphonoacetyl)-L-aspartate.

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