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Characterization of the Plant Nicotinamide Adenine Dinucleotide Kinase Activator Protein and Its Identification as Calmodulin†

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ABSTRACT: A protein activator of plant NAD kinase has been extracted from plant sources (peanuts and peas), purified to homogeneity, characterized, and identified as calmodulin. A comparison of the properties of calmodulin isolated from either plant or animal sources shows that they are strikingly similar proteins. The similarities include molecular weight, Stokes radii, amino acid composition, Ca^{2+}-dependent enhancement of tyrosine fluorescence, Ca^{2+}-dependent interaction with troponin I, equal abilities to activate cyclic nucleotide phosphodiesterase, Ca^{2+}-dependent inhibition of calmodulin action by the phenothiazine drugs, and electrophoretic mobility. We discuss the possibility that plant cells may undergo Ca^{2+}-dependent regulatory events that are mediated by calmodulin in a manner similar to those found in animals.

Calmodulin, a low molecular weight Ca^{2+}-binding protein, has been isolated from a variety of animal sources including coelenterates, annelids, and mammals (Teo et al., 1973; Teo & Wang, 1973; Lin et al., 1974; Wolff & Brostrom, 1974; Childers & Siegel, 1975; Dedman et al., 1977a; Yagi et al., 1978; Dabrowska et al., 1978; Waisman et al., 1978a; Jones et al., 1979; Head et al., 1979). Similarities in the physicochemical properties of calmodulin isolated from these sources, along with recent sequence data (Watterson et al., 1976; Vanaman et al., 1977; Dedman et al., 1978), suggest that the primary structure of calmodulin has been highly conserved during evolution. A high degree of conservation has been shown recently by a comparison of the amino acid sequences of coelenterate and mammalian calmodulin (F. Sharief, H. P. Jones, M. J. Cormier, and T. C. Vanaman, unpublished experiments). Such data suggest one or more fundamental roles for calmodulin in Ca^{2+}-dependent regulatory processes. Indeed, calmodulin has been found to activate a number of enzymes in vitro, including cyclic nucleotide phosphodiesterase (Cheung, 1970; Kakiuchi et al., 1970), brain adenylate cyclase (Brostrom et al., 1975; Cheung et al., 1975), (Ca^{2+} + Mg^{2+})-ATPase (Jarrett & Penniston, 1977, 1978; Gopinath & Vincenzi, 1977), several protein kinases (Yagi et al., 1978; Dabrowska et al., 1978; Waisman et al., 1978a,b), plant NAD kinase (Anderson & Cormier, 1978), phosphorylase kinase (Cohen et al., 1978), and phospholipase A_2 (Wong & Cheung, 1979). Calmodulin has also been found to be involved in regulating cellular processes such as the phosphorylation of synaptic vesicle proteins with subsequent release of neurotransmitter (Schulman & Greengard, 1978; DeLorenzo et al., 1979) and the disassembly of microtubules (Marcum et al., 1978).

Preliminary studies in this laboratory have shown that a heat-stable, Ca^{2+}-dependent activator of partially purified plant NAD kinase exists in extracts of higher plants, and that this activator protein has many properties in common with those observed for calmodulin isolated from animal sources (Anderson & Cormier, 1978). It was also shown that mammalian brain calmodulin will replace the plant protein activator in the Ca^{2+}-dependent activation of this plant NAD^+ kinase preparation. We report here the characteristics of this plant ac-

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tivator protein and its identity with calmodulin. The significance of the presence of calmodulin in plants is discussed.

Materials and Methods

Calmodulin was purified from extracts of peanut seeds (*Arachis hypogea*), pea seedlings (*Pisum satavum* L. cv. Willet Wonder), a coelenterate (*Renilla reniformis*), and porcine brain by use of fluphenazine–Sepharose affinity chromatography as previously described (Charbonneau & Cormier, 1979). We have since found that the use of acetone powder preparations of peanut seeds facilitates the purification procedure.

Troponin I, troponin C, and bovine brain calmodulin were gifts from Dr. T. C. Vanaman, Duke University. Trifluoperazine was a gift from Smith Kline and French Laboratories; fluphenazine was a gift from the Squibb Institute; and trimethyllysine was a gift from Dr. Mary Anne Conti, National Institutes of Health. All reagents used were of the best grades commercially available.

Partially purified preparations of NAD kinase were obtained from pea seedlings grown for 10 days under fluorescent illumination at 25 °C. Plants were cut above the seed and homogenized in 1.0 M KCl, 50 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, and 1 mM EGTA (buffer A) containing 2.5% w/v poly(vinylpyrrolidone) and 0.5 mM phenylmethanesulfonyl fluoride at 4 °C. One gram of tissue was homogenized in 4 mL of buffer A using first a Tekmar SD 45 homogenizer followed by a Willems Polytron PT-20St for 3 min. KCl was added to this homogenate to a final concentration of 1.0 M, and then the homogenate was filtered through two layers of cheesecloth followed by centrifugation at 9000*g* for 30 min. The resultant supernatant was diluted 1:1 with deionized water, loaded onto a DEAE-cellulose column, and eluted with buffer A, diluted 1:1 with deionized water. Ammonium sulfate was then added to the eluant to a final concentration of 50%, and the suspension was centrifuged at 9000*g* for 20 min. The resultant pellet was resuspended in a minimum volume of buffer A (diluted 1:9 with deionized water) and dialyzed against the same buffer. The dialysate was loaded onto a second DEAE-cellulose column and eluted with buffer A diluted 10-fold with deionized water. The presence of 1.0 M KCl in the extraction buffer increased enzyme yields substantially over those of the procedure previously reported (Anderson & Cormier, 1978), and the maintenance of 0.1 M KCl in solutions throughout the purification procedure further minimized enzyme losses.

Assays. Calmodulin was assayed on the basis of its ability to stimulate the activity of calmodulin-dependent porcine brain phosphodiesterase using the firefly bioluminescence assay described by Matthews & Cormier (1978) with the following modifications: (a) The phosphodiesterase reaction was run in 90 μL of buffer A (Matthews & Cormier, 1978) plus 10 μL of calmodulin sample; the reaction was initiated with 5 μL of phosphodiesterase solution. (b) The phosphodiesterase reaction was incubated for 10 min at room temperature and stopped by immersion in a boiling water bath for 5 min. (c) The AMP produced in the phosphodiesterase reaction was converted to ATP by adding 100 μL of solution 2 (Matthews & Cormier, 1978) to the cooled reaction mixture and incubating at room temperature for 1 h before assay. One unit of calmodulin was defined as the quantity of protein giving 50% maximal stimulation of calmodulin-dependent phosphodiesterase. In the absence of calmodulin, the phosphodiesterase solution (pH 8.0) hydrolyzed 40 pmol of cAMP per min at room temperature. The addition of calmodulin resulted in a fourfold enhancement of phosphodiesterase activity. Calmodulin-dependent phosphodiesterase was isolated from porcine brain by the procedure of Egrie (1975).

NAD kinase assays were performed at 37 °C in a final volume of 0.5 mL containing 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 30 mM KCl, 0.15 mL of partially purified NAD kinase, 25 μg of plant calmodulin, and either 0.1 mM Ca^{2+} or 0.1 mM EGTA. The reaction was allowed to proceed for 30 min and was stopped by heating at 85 °C for 3 min, and the precipitate was removed by centrifugation. The volume was adjusted to 1 mL by the addition of 100 mM Tris-HCl, pH 8.0, containing 0.2 mM EGTA. The remainder of the assay was performed as previously described (Muto & Miyachi, 1977).

Luminescence and Spectral Measurements. Light measurements were made on a photometer that is capable of simultaneously measuring the initial peak intensity and total integrated light of the bioluminescence reaction (Anderson et al., 1978). The spectral measurements reported here were obtained on a previously described online computer-controlled spectrophotometer (DeSa & Wampler, 1973).

Physical Measurements. Analytical ultracentrifugation was carried out in a Beckman-Spinco Model E ultracentrifuge equipped with absorption optics and a photoelectric scanning device. All ultracentrifugation experiments were performed with calmodulin samples that had been extensively dialyzed against 10 mM Tris-HCl and 0.2 M NaCl, pH 8.0. Sedimentation velocity studies were done at 20 °C using schlieren optics and a protein concentration of 2.0 mg/mL. The molecular weight of calmodulin was calculated from sedimentation equilibrium runs using the low-speed method (13 000 rpm) with absorption optics. The partial specific volume of the protein was calculated from its amino acid composition by the procedure of Cohn & Edsall (1943). The extinction coefficient of plant calmodulin was determined using a calmodulin solution of known absorbance and measuring the protein concentration by ultracentrifugation using interference optics (Babul & Stellwagon, 1969). For these measurements calmodulin was dissolved in 10 mM Tris-HCl, pH 8.0.

Polyacrylamide Gel Electrophoresis. Analytical NaDodSO_4 gel electrophoresis was conducted according to the procedure of Laemmli (1970) on 15% polyacrylamide slab gels in the presence of 1.5 mM EGTA. Standards and plant calmodulin were prepared for electrophoresis by heating at 95 °C for 5 min in the presence of 1% NaDodSO_4, 1% β-mercaptoethanol, and 15 mM EGTA. Ovalbumin, bovine erythrocyte carbonic anhydrase, whale skeletal myoglobin, and horse heart cytochrome *c* were employed as standards. The data was plotted as log R_f vs. molecular weight.

Plant calmodulin was compared with troponin C and bovine brain calmodulin for its ability to complex with troponin I based on the polyacrylamide gel electrophoretic method of Amphlett et al. (1976).

Ca^{2+} Enhancement of Tyrosine Fluorescence. Tyrosine fluorescence measurements were performed at room temperature in a Perkin-Elmer MPF 44 spectrofluorimeter. Tyrosine emission intensity was measured at 310 nm with excitation at 276 nm. The dependence of tyrosine fluorescence on Ca^{2+} was studied using a Ca^{2+}-EGTA buffer system containing 50 mM Tris, 100 mM KCl, and 1 mM EGTA (pH 7.89). Protein solutions were prepared for these measurements by preliminary dialysis against 50 mM Tris and 5 mM EGTA, pH 8.0, followed by a final extensive dialysis against the EGTA buffer described above. The absorbance of all protein samples was less than 0.040 at 276 and 310 nm. The titrations were performed by adding 1- to 20-μL aliquots of a Ca^{2+} standard

Table I: Properties of Calmodulin Isolated from Plant and Animal Sources

property	source of calmodulin		
	plant	coelenterate (*Renilla*)[a]	mammalian
Stokes radius (Å)	22	22	23[b]
$s_{20,w}$	1.9	1.5	1.9[b]
molecular weight			
from $NaDodSO_4$ gel electrophoresis	17 300	15 600	18 000[c]
from gel filtration (K_D vs. M_r)	23 300	25 000	28 200[b]
from sedimentation equilibrium	14 600	18 750	17 800[b]
extinction coeff, $\epsilon_{276}^{1\%}$	0.9	1.0[d]	1.8[c]
Phe/Tyr ratio	8	9[e]	4[c]
absorption maxima (nm)	279, 268, 265, 259, 253	276, 268, 264, 258, 252	277, 269, 265, 259, 253[f]
Ca^{2+}-dependent enhancement of Tyr fluorescence	+		+[b]
Ca^{2+}-dependent interaction with Troponin I	+	+	+[g]
amount required for 50% activation of phosphodiesterase (ng)[h]	9		8
stelazine inhibition of phosphodiesterase act.	+	+	+[i]
activation of plant NAD kinase	+	+	+

[a] Data from Jones et al. (1979) except where indicated. [b] Dedman et al. (1977a). [c] Watterson et al. (1976). [d] Our previously reported value of 1.9 was based on a biuret measurement of protein which gives an artificially low protein value for *Renilla* calmodulin. [e] By sequence (Sharief et al., manuscript in preparation). [f] Cheung et al. (1978). [g] Amphlett et al. (1976). [h] Under the assay conditions phosphodiesterase catalyzed the conversion of 40 pmol of cAMP min^{-1} in the absence of calmodulin (see Materials and Methods). [i] Levin & Weiss (1977).

solution to a 3.0-mL solution of protein in a fluorescence cuvette. The Ca^{2+} standards were prepared by making the appropriate dilutions of a Ca^{2+} standard solution obtained from Orion. During the titration, the pH was held at 7.89 by adding the appropriate volumes of 3% KOH when necessary. Ca^{2+} and KOH additions were made with a Hamilton CR-700-20 spring-loaded automatic constant speed syringe, which was previously calibrated by radioactivity measurements. Fluorescence intensities were corrected for dilutions occurring during the titration.

Free Ca^{2+} concentrations in the Ca^{2+}-EGTA buffer system were calculated on a digital computer using the Newton–Raphson method of successive approximation to solve the appropriate equation for the free Ca^{2+} concentration. The equilibrium constants for the binding of Ca^{2+} and H^+ to EGTA were taken from Potter & Gergely (1975). Since our buffers contained <1 μM Ca^{2+}, no corrections were made for this level of contamination. These measurements were made with a Perkin-Elmer Model 306 atomic absorption spectrometer equipped with an HGA 2200 graphite furnace. The values used for the total calcium and EGTA concentrations were corrected for the total volume change occurring on the addition of Ca^{2+} and KOH solutions.

Chemical Analyses. All amino acid analyses were performed on a Beckman Model 119 C amino acid analyzer. Samples containing 30 μg of protein were hydrolyzed in 6 N HCl at 110 °C in sealed evacuated tubes for 24, 48, and 72 h. A known quantity of internal standard, norleucine, was added to the samples prior to hydrolysis. Most amino acids were determined as the average of 24-, 48-, and 72-h hydrolysates of calmodulin. For composition analysis, time courses of hydrolysis were analyzed to correct for destructive losses of serine and threonine and for the slow release of valine and isoleucine. Cysteine was determined as cysteic acid by amino acid analysis of 24-h hydrolysates of performic acid oxidized protein (Hirs, 1967). Tryptophan was determined by amino acid analysis of samples hydrolyzed for 24 h in the presence of 4% thioglycolic acid (Matsubara & Sasaki, 1969). N^{ϵ}-Trimethyllysine was determined independently, following a 24-h hydrolysis, by using a modification of the standard Beckman program as follows: the pH of each buffer system was adjusted to 3.2, 4.1, and 5.4 with program times of 68, 32, and 130 min, respectively. Trimethyllysine eluted 6 min after ammonia and 3 min before lysine using this modification.

Carbohydrate analyses were performed by loading 75 μg of plant calmodulin along with the glycoprotein standards, α-1-glycoprotein, and γ-globulin onto 15% gels according to the method of Gabriel (1971). The gels were stained for carbohydrate according to the method of Segrest & Jackson (1977).

Results

Recent preliminary reports have described the rapid isolation of calmodulin from a wide variety of sources by the use of phenothiazine–Sepharose affinity chromatography (Charbonneau & Cormier, 1979; Jamieson & Vanaman, 1979). Plant calmodulin utilized in these studies was purified by chromatography on fluphenazine–Sepharose as previously described (Charbonneau & Cormier, 1979). The chemical, physical, and biological characteristics of plant calmodulin were determined and compared with those of calmodulin isolated from animal sources. Calmodulin used in this study was isolated from peanut seeds and is referred to here as plant calmodulin. However, we have also obtained a highly purified preparation of calmodulin from pea seedlings which has similar chemical and biological properties.

Physicochemical Data. A number of physical and biological properties of plant calmodulin are summarized in Table I. In each case the property listed is compared to calmodulin isolated from a mammal and an anthozoan coelenterate.

The molecular weight of native plant calmodulin was determined by low-speed sedimentation equilibrium studies. Linear plots of ln c vs. r^2 were obtained, resulting in a calculated molecular weight of 14 600. $NaDodSO_4$ gel electrophoresis of plant calmodulin on 15% polyacrylamide gels resulted in a single protein band which corresponded to a molecular weight of 17 300 when compared to appropriate standards (see Materials and Methods). As shown in Figure 1, plant calmodulin appears to have a lower molecular weight than bovine brain calmodulin from $NaDodSO_4$ gel data. This difference may be more apparent than real. For example, the $NaDodSO_4$ molecular weights for *Renilla* and bovine brain

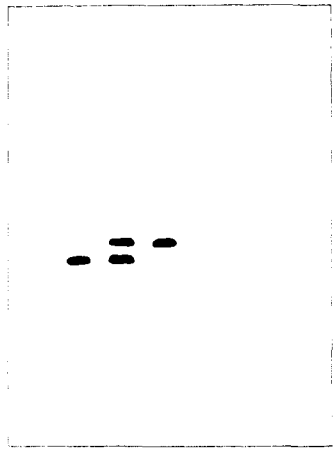

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of plant and bovine calmodulin. Wells were loaded with 10 μg of each protein: (A) plant calmodulin; (B) plant and bovine brain calmodulin; (C) bovine brain calmodulin.

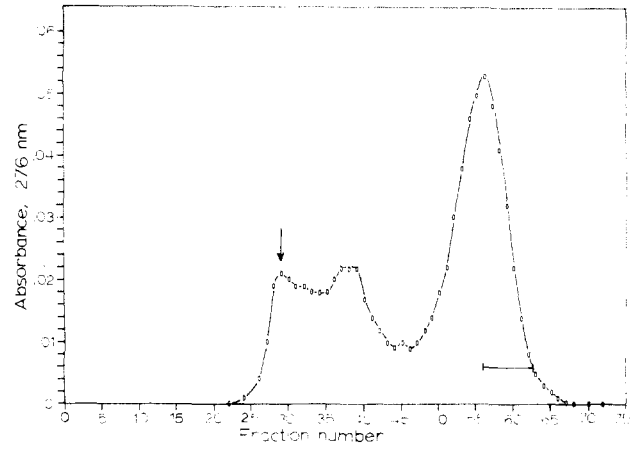

FIGURE 2: Gel filtration chromatography of a homogeneous preparation of plant calmodulin. The column (3.8 × 66 cm) was equilibrated with 50 mM Tris-HCl and 5 mM $CaCl_2$, pH 8.0. Four milliliter fractions were collected, and their absorbances monitored, after loading a sample volume of 8 mL at a protein concentration of 2.5 mg/mL.

were reported as 15 600 and 18 000, respectively (Jones et al., 1979), whereas the molecular weights of these two proteins are actually similar as shown by recent amino acid sequence data (F. Sharief, H. P. Jones, M. J. Cormier, and T. C. Vanaman, unpublished experiments). In addition, bovine brain and plant calmodulin showed indistinguishable mobilities on an $NaDodSO_4$–urea–polyacrylamide gel system described by Swank & Munkres (1971). Thus, the data show that plant calmodulin is a single polypeptide chain whose molecular weight is similar to that of animal calmodulin.

The Stokes radius of plant calmodulin was determined by gel filtration on Sephadex G-75 superfine equilibrated with 10 mM Tris and 200 mM KCl, pH 8.0. Ovalbumin, soybean trypsin inhibitor, horse heart cytochrome *c*, and whale skeletal myoglobin were used as standards with blue dextran and thiamin included as markers of the excluded and included volume of the column. Samples and standards were chromatographed in triplicate, and the data, which was plotted as $K_D^{1/3}$ vs. Stokes radius, resulted in a linear plot with a correlation coefficient of 0.98 (Porath, 1963). The Stokes radius for plant calmodulin determined by this method was 22 ± 0.1 Å in either the presence of 5 mM EGTA or 1 mM $CaCl_2$. As shown in Table I, similar values have been reported for calmodulin isolated from both coelenterate and mammalian sources. Similar values for $S_{20,w}$ are also observed regardless of the source of calmodulin.

Homogeneous preparations of calmodulin from all sources examined have shown light scattering in their absorption spectra. Such light scattering could be caused by aggregation. In fact, a high molecular weight component was observed in some of our earlier sedimentation equilibrium runs on plant calmodulin. The formation of aggregates appears to occur upon storing solutions of plant calmodulin. Gel filtration of such a solution on Sephadex G-75 superfine provides a direct demonstration of the formation of aggregates (Figure 2). The calmodulin preparation used in this experiment showed a single protein band on $NaDodSO_4$ gel electrophoresis corresponding to a molecular weight position of 17 300. However, the aggregates could represent a relatively small percentage of the total protein, since light scattering may significantly increase the absorbance at 276 nm (Figure 2). Aggregate-free calmodulin eluted in fractions 56–63 (Figure 2), while the arrow indicates the position of the void volume. It is apparent from Figure 2 that higher molecular weight forms of calmodulin existed in our preparations. For all physical measurements and biological activity determinations, plant calmodulin fractions corresponding to the monomeric form of the protein were used as indicated by the bar in Figure 2.

Table II: Amino Acid Compositions of Calmodulin Isolated from Plant and Animal Sources

	plant (peanut)[a]		mammalian (bovine brain)[b]	coelenterate (*R. reniformis*)[c]
	av	nearest integer		
Lys	7.9	8	7	8
His	1.2	1	1	1
Me_3Lys	1.6	1–2	1	1
Arg	4.4	4	6	6
Asp	27.1	27	23	23
Thr	9.1	9	12	12
Ser	5.3	5	4	5
Glu	28.2	28	27	26
Pro	2.3	2	2	2
Gly	11.2	11	11	11
Ala	10.6	11	11	10
Cys		1[d]	0	0
Val	6.3	6	7	7
Met	7.3	7	9	8
Ile	5.9	6	8	8
Leu	11.2	11	9	9
Tyr	0.6	1	2	1
Phe	8.2	8	8	9
Trp	0	0	0	0

[a] Determined as described in the text. Values expressed as moles of amino acid per 16 700 g of protein. [b] Based on the amino sequence of Vanaman et al. (1977). [c] Based on the amino acid sequence of Sharief et al. (manuscript in preparation). [d] One residue of cysteine was observed in samples dissolved in 6 M guanidine hydrochloride and reduced with 1 mM dithiothreitol (Dr. T. C. Vanaman, personal communication). This observation has been confirmed in this laboratory by performic acid oxidation.

Chemical Data. As shown in Table II, the amino acid compositions of calmodulin isolated from plant and animal sources are remarkably similar. We used an M_r of 16 700 for plant calmodulin for calculation of the amino acid composition, although some physical measurements suggest a somewhat lower value (see Table I). As the above discussion of Figure 1 indicates, some of these differences in molecular weight with different techniques may be more apparent than real. The similarities in amino acid composition include the presence of trimethyllysine, a relative preponderance of negatively charged amino acids, two proline residues, and the lack of tryptophan.

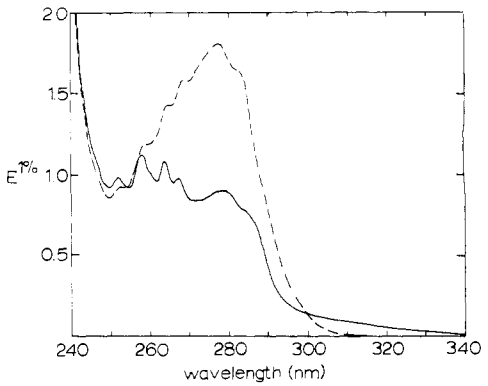

FIGURE 3: A comparison of the absorption spectra of plant (—) and porcine brain (---) calmodulin.

The lack of tryptophan in plant calmodulin is also indicated by its absorption spectrum (Figure 3) and the lack of tryptophan fluorescence. Of interest is the presence of one residue of cysteine in plant calmodulin. Consistent with the findings for other calmodulins, no carbohydrate was detected in the purified preparations of plant calmodulin.

A significant difference between plant and bovine brain calmodulin is seen in their phenylalanine to tyrosine ratios (Table II). Plant calmodulin contains one tyrosyl residue, but mammalian calmodulin contains two such residues (Vanaman et al., 1977; Klee, 1977); both plant and mammalian calmodulins contain eight phenylalanine residues. The presence of one tyrosine in plant calmodulin is confirmed by the $\epsilon_{276}^{1\%}$ of 0.9 as compared with an $\epsilon_{276}^{1\%}$ of 1.8 for mammalian calmodulin. As shown in Figure 3, calmodulin isolated from either plant or mammalian sources exhibits multiple absorption maxima at 276–279, 268, 265, 258, and 253 nm. The difference in absorbance at 279 nm is consistent with the presence of one tyrosyl residue in plant calmodulin and two in mammalian calmodulin. The presence of a single tyrosyl residue in calmodulin isolated from the marine coelenterate, *Renilla reniformis*, has recently been shown by amino acid sequence data (F. Sharief, H. P. Jones, M. J. Cormier, and T. C. Vanaman, unpublished experiments). It appears that plant calmodulin represents a second such example.

Using $NaDodSO_4$–urea–polyacrylamide gel electrophoresis (Swank & Munkres, 1971), we found that all peptides obtained by cyanogen bromide digestion of calmodulins isolated from bovine brain and human erythrocytes showed identical patterns. Only two peptides derived from plant calmodulin had mobilities which were identical with peptides from the mammalian sources (H. W. Jarrett et al., unpublished experiments).

Ca^{2+} Enhancement of Tyrosine Fluorescence. Recent studies have clearly shown that calmodulin undergoes a conformational transition upon the binding of Ca^{2+} (Wang et al., 1975; Liu & Cheung, 1976; Klee, 1977; Wolff et al., 1977; Dedman et al., 1977a; Richman & Klee, 1979). Fluorescence, absorption spectra, and chemical modification studies indicate that a change in the microenvironment of the tyrosine residues accompanies the Ca^{2+}-induced conformational transition (Wang et al., 1975; Dedman et al., 1977a; Wolff et al., 1977; Klee, 1977; Richman & Klee, 1979). Dedman et al. (1977a) have studied the effects of Ca^{2+} on the tyrosine fluorescence of rat testis calmodulin. As the Ca^{2+} concentration increased from approximately 10^{-7} to 10^{-5} M, a 2.5-fold enhancement in tyrosine fluorescence was measured.

We have utilized tyrosine fluorescence measurements to examine the effects of Ca^{2+} binding on plant calmodulin. The Ca^{2+} dependence of tyrosine fluorescence was examined using

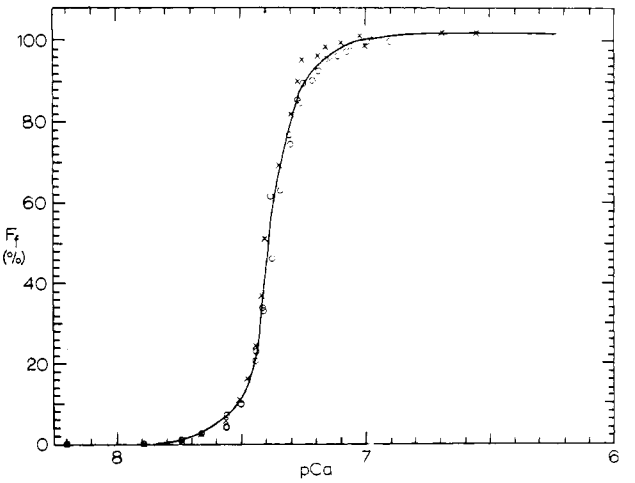

FIGURE 4: Effect of Ca^{2+} on the tyrosine fluorescence of plant and porcine brain calmodulin. A Ca^{2+}-EGTA buffer solution (50 mM Tris, 100 mM KCl, and 1 mM EGTA, pH 7.89) was used to produce the desired free Ca^{2+} concentrations. The free Ca^{2+} concentration was calculated as described under Materials and Methods. The fluorescence measurements were recorded at 305 nm at room temperature as described under Materials and Methods. Samples of plant (O–O) and porcine brain (×–×) calmodulin contained 0.24 and 0.23 mg/mL protein, respectively. Protein concentration was determined by absorbance measurements.

both plant and porcine brain calmodulin at nearly identical concentrations (Figure 4). An increase in the Ca^{2+} concentration from approximately 10^{-8} to 10^{-7} M resulted in 2.5- and 2.4-fold enhancement of tyrosine fluorescence for porcine brain and plant calmodulin, respectively. This 2.4-fold increase in the tyrosine fluorescence intensity suggests that plant calmodulin undergoes a conformational transition resulting in a change in the microenvironment of the single tyrosine residue which is similar to that observed with mammalian calmodulin. As shown in Figure 4, equal concentrations of plant and porcine brain calmodulin show nearly identical Ca^{2+} titration curves with 50% of full enhancement occurring at 4.1×10^{-8} M Ca^{2+}. This observation suggests that the Ca^{2+} binding parameters of plant calmodulin are similar to those of mammalian calmodulin. Dedman et al. (1977a) observed an increase in tyrosine fluorescence occurring over a range of Ca^{2+} concentrations which are about 10-fold higher than those measured in this study. This difference could be due to differences in buffers and pH used in these measurements. Our measurements were performed in 50 mM Tris, 100 mM KCl, and 1 mM EGTA, pH 7.89, while Dedman et al. (1977a) used 90 mM KCl, 10 mM K_2HPO_4, and 2 mM EGTA, pH 7.0. The differences observed cannot be attributed to reagent contamination by Ca^{2+}, since total Ca^{2+} levels in our buffers and KOH solutions were found to be <1 μM (see Materials and Methods).

Richman & Klee (1978, 1979) have shown that the two tyrosine residues (99 and 138) of mammalian calmodulin are in different microenvironments and behave differently when Ca^{2+} is bound to the protein. Ca^{2+} binding affects only the microenvironment of tyrosyl residue 138. It is noteworthy that amino acid composition and spectral data show that plant calmodulin contains only one tyrosine residue. The observation that plant calmodulin shows a 2.4-fold enhancement in tyrosine fluorescence is consistent with the data of Richman & Klee (1978, 1979) and shows that a single tyrosyl residue can account for a large increase in tyrosine fluorescence upon the binding of Ca^{2+}. These observations suggest the possibility that the single tyrosyl residue of plant calmodulin is located at or near position 138. Indeed, recent sequence data have

shown that *Renilla* calmodulin contains only one tyrosine residue located at position 137 (F. Scharief, H. P. Jones, M. J. Cormier, and T. C. Vanaman, unpublished experiments).

Biological Activities of Plant Calmodulin. Plant and porcine brain calmodulin are equally active in stimulating calmodulin-dependent porcine brain cyclic nucleotide phosphodiesterase. A comparison of the concentration dependence of these two calmodulins on the activation of phosphodiesterase showed that the amount of calmodulin required for 50% maximal activation of identical amounts of the enzyme was approximately the same in each case (~8 ng; see Table I). In both cases three- to fourfold stimulation of phosphodiesterase was observed when saturating amounts of calmodulin were included in the assay. Plant calmodulin, as well as mammalian calmodulin, requires Ca^{2+} in order to stimulate the activity of calmodulin-dependent phosphodiesterase. It has been shown that the Ca^{2+}-dependent binding of phenothiazine drugs, such as trifluoroperazine, to bovine brain calmodulin will inhibit its ability to activate a number of enzymes such as calmodulin-dependent phosphodiesterase (Levin & Weiss, 1977; Weiss & Levin, 1978; Brostrom et al., 1978). This inhibition is due to the binding of trifluoperazine to two Ca^{2+}-dependent high-affinity sites (K_d = 1 μM) on calmodulin and to ~24 low-affinity Ca^{2+}-independent sites (K_d = 5 mM; Weiss & Levin, 1978). An examination of the effects of these drugs on plant calmodulin showed that the activation of porcine brain phosphodiesterase by both plant and bovine brain calmodulin was completely inhibited by 0.1 mM trifluoperazine (Table I). In addition, we have also shown that plant calmodulin will bind to fluphenazine–Sepharose in a Ca^{2}-dependent manner (Charbonneau & Cormier, 1979). These observations suggest that plant calmodulin, like mammalian calmodulin, possesses Ca^{2+}-dependent phenothiazine binding sites.

The activity of a partially purified pea NAD kinase preparation, which formed 0.9 nmol of NADP min^{-1} in the presence of 0.1 mM $CaCl_2$, was stimulated about sevenfold upon the addition of 25 μg of plant calmodulin in a final volume of 0.5 mL. In some preparations of NAD kinase the stimulation by calmodulin was as much as 30-fold. This variation in activity enhancement by calmodulin may reflect varying degrees of calmodulin contamination in the NAD kinase preparations. The calmodulin-dependent stimulation of NAD kinase is also Ca^{2+} dependent, since no stimulation by plant calmodulin was observed when 0.1 mM $CaCl_2$ was replaced by 0.1 mM EGTA in the assay. In the presence of 0.1 mM trifluoperazine, the addition of calmodulin plus Ca^{2+} provided no stimulation of NAD^+ kinase activity. The ability of trifluoperazine to prevent the stimulation of NAD kinase activity by calmodulin appears to be analogous to the effects of this drug on the calmodulin-dependent activation of other enzymes such as cyclic nucleotide phosphodiesterase and brain adenylate cyclase [see Levin & Weiss (1979) for a review].

A recent comparison was made of the Ca^{2+} concentration dependence for the activation of both NAD kinase and porcine brain phosphodiesterase by plant calmodulin (Anderson & Cormier, 1978). The Ca^{2+} dependence of each was similar, showing that plant calmodulin, like mammalian calmodulin, will respond to micromolar levels of free Ca^{2+}.

An additional similarity between plant and mammalian calmodulins is apparent in their abilities to form hybrid complexes with troponin I. Calmodulin isolated from mammalian sources will replace troponin C to form a hybrid protein–protein complex with troponin I in the presence of Ca^{2+} (Amphlett et al., 1976; Dedman et al., 1977b). Such complexes are easily observed by using polyacrylamide gel electrophoresis techniques (Amphlett et al., 1976). Using such procedures we have found that plant and bovine brain calmodulin will both form Ca^{2+}-dependent calmodulin–troponin I complexes that are electrophoretically similar to the native troponin C–troponin I complex.

Finally, homogeneous preparations of plant calmodulin, prepared in this laboratory, were analyzed for their immunological cross-reactivity relative to rat testis [^{125}I]calmodulin in a radioimmunoassay (Chafouleas et al., 1979). Calmodulin from both sources was found to compete in an identical manner in the assay.

Discussion

A comparison of the properties of calmodulin isolated from either plant or animal sources shows that they are strikingly similar proteins. The similarities include (a) molecular weight, (b) Stokes radii, (c) amino acid composition, (d) Ca^{2+}-dependent enhancement of tyrosine fluorescence, (e) Ca^{2+}-dependent interaction with troponin I, (f) equal abilities to activate cyclic nucleotide phosphodiesterase, (g) Ca^{2+}-dependent inhibition of calmodulin action by the phenothiazine drugs, (h) electrophoretic mobility during both polyacrylamide disc and $NaDodSO_4$–polyacrylamide gel electrophoresis, and (i) immunological cross-reactivities. One significant difference is the presence of a single cysteine residue in plant calmodulin which may be responsible for the tendency of this protein to form aggregates. In fact, the extent of the similarities between calmodulins isolated from eucaryotic organisms as evolutionarily distant as peanuts and pigs supports the idea that this protein plays a vital role in eucaryotic life. Therefore, the presence of calmodulin in plants suggests that plant cells may undergo Ca^{2+}-dependent regulatory events that are mediated by calmodulin in a manner similar to those found in animals.

Calmodulin may play a role in stimulus–response coupling in animals, in which a Ca–calmodulin complex may act as a second messenger in a manner analogous to the second messenger role of cAMP. In the unstimulated cell, the free Ca^{2+} concentration [10^{-7}–10^{-8} M; see Blinks et al. (1976) for a review] is too low to bind to calmodulin, since the concentration is considerably below the K_d value of approximately 10^{-6} M (Vanaman et al., 1977, Dedman et al., 1977a). In this state the calmodulin-dependent enzymes are turned "off". Upon stimulation, the cytosolic free Ca^{2+} concentration rises to a level (10^{-4}–10^{-5} M) considerably above the K_d value for Ca^{2+} binding to calmodulin. In this state Ca^{2+} binds to calmodulin forming a complex which in turn binds to calmodulin-dependent enzymes turning them "on". Presumably, during relaxation Ca^{2+} is pumped out of the cell or rapidly resequestered. This results in dissociation of the calmodulin–enzyme complexes which turns them "off". In vitro evidence for this mechanism of calmodulin-dependent regulation has been obtained in several laboratories (Watterson & Vanaman, 1976; Lynch et al., 1976; Ho et al., 1977; Cheung et al., 1978; Lynch & Cheung, 1979; Nairn & Perry, 1979). It is thus possible that Ca^{2+}, acting through calmodulin, may be involved in regulatory events that can be either rapid, as one would expect from a Ca^{2+} transient, or long-lived, as one would expect from a sustained intracellular Ca^{2+} concentration gradient.

Support for the idea that calmodulin plays a similar role in plants comes from the observation that calmodulin stimulates pea NAD kinase in a Ca^{2+}-dependent manner. The relative levels of NAD and NADP have regulatory significance in the plant cell, and NAD has been shown to be converted to NADP upon illumination of plant leaves (Krogman & Ogren, 1965). Taken together, the data suggest that the

stimulus (light) may activate NAD kinase via calmodulin, with Ca^{2+} being a necessary link in the process.

In addition, there is increasing evidence that Ca^{2+} is important in the regulation of a number of physiological processes in plants. For example, red light has been shown to stimulate the rotation of the chloroplast of the filamentous green alga, *Mougeotia* (Haupt, 1959). The rotation does not occur in far-red light or in the absence of Ca^{2+} in the medium. Red light illumination of *Mougeotia* also results in the intracellular accumulation of Ca^{2+} (Dreyer & Weisenseel, 1979). This observation led to the suggestion that Ca^{2+} may be a common messenger in the numerous physiological events controlled by phytochrome.

Phytochrome has also been shown to be involved in the depolarization of *Nitella* cells, and again the depolarization is dependent on external Ca^{2+} (Weisenseel & Ruppert, 1977). It is also of interest that cytoplasmic streaming in *Nitella* is inhibited by Ca^{2+} concentrations above 10^{-7} M (Hayama et al., 1979).

Furthermore, Ca^{2+} also appears to play a role in the regulation of directional growth. Using eggs of the brown alga, *Fucus pelvetia*, it has been observed that unilateral light establishes an ion current that passes through the cell and originates from the point of stimulus (Jaffe, 1979). One of the major components of this ion current is Ca^{2+}, and, since Ca^{2+} has a low effective mobility in cells, the result is the generation of a Ca^{2+} concentration gradient across the cytosol. That such a Ca^{2+} concentration gradient can form has been demonstrated in experiments employing $^{45}Ca^{2+}$ and low-temperature autoradiography (Jaffe et al., 1975; Jaffe, 1979). In these experiments, Ca^{2+} was found to be concentrated about threefold within the cytosol of the growing tip relative to opposite regions of the cell. In addition, the growing tip of pollen tubes has also been found to contain elevated levels of Ca^{2+} relative to other regions of the cell. Growth of the tube is dependent on an external supply of Ca^{2+} and appears to be directed by an intracellular Ca^{2+} gradient (Wisenseel & Jaffe, 1976; Herth, 1978; Reiss & Herth, 1978, 1979a). If this gradient is disrupted by treatment of the pollen tube with the Ca^{2+} ionophore A23187, which would allow Ca^{2+} entry at other than the tip, then growth ceases and the vesicular organization at the tip becomes disrupted. Thus, not only is Ca^{2+} required for directional growth, but Ca^{2+} must also enter at the tip of the growing tube. Recently, Reiss & Herth (1979b) have also shown that Ca^{2+} gradients occur in the growing tips of plant root hairs, fungal mycelium, moss caulonema, and *Acetabularia* stalks and whorls. As Kretsinger (1977) has pointed out, there are no known cellular components other than Ca^{2+}-binding proteins which can bind Ca^{2+} at concentrations found in the cytosol of stimulated cells, i.e., $\sim 10^{-5}$ M. We suggest that the target for Ca^{2+} in plant cells, like animal cells, is most probably a Ca^{2+}-binding protein such as calmodulin. Thus, calmodulin may play a role in Ca^{2}-dependent regulatory events in both plant and animal cells.

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