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CALCIUM-DEPENDENT REGULATOR OF NAD KINASE IN HIGHER PLANTS

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SUMMARY. An activator protein of NAD kinase from the pea, Pisum satavum L., has been shown to be Ca^{2+}-dependent. This plant activator protein also stimulates the activity of modulator protein dependent-cyclic nucleotide phosphodiesterase from porcine brain. This stimulation is similar to that observed with modulator protein isolated from animal sources. Furthermore, Ca^{2+}-dependent modulator proteins isolated from porcine brain, bovine brain, and the coelenterate, Renilla, will regulate the NAD kinase activity of peas. Other common properties of the plant activator protein and animal modulator proteins are their acidic nature, heat stabilities, similar Stokes' radii, and their interactions with troponin I.

In many types of animal cells the intracellular Ca^{2+} concentration is important in the regulation of a number of cellular events such as microtubule assembly (1-3), stimulus-secretion coupling (4-5), the Ca^{2+} pump of the erythrocyte membrane (6-7), the contractile-relaxation cycle (8-11), egg fertilization, (12,13) and the levels of cytoplasmic cyclic nucleotides (14-20). The individual event is controlled by a transient increase in the cytoplasmic Ca^{2+} concentration from an unstimulated level of approximately 10^{-7}M to a stimulated value of approximately 10^{-5}M (21). A Ca^{2+} binding protein, which has been isolated from a variety of animal tissues, has been implicated in the regulation of many of the above processes. This protein, termed modulator protein or a Ca^{2+}-dependent regulator, was originally discovered as a protein activator of cyclic nucleotide phosphodiesterase (14-16). The presence of high affinity Ca^{2+} binding sites (22-26) with a K_d in the μM range allows this class of proteins to act as a sensor of rapid Ca^{2+} transients. It has been shown that the functional species is a modulator protein complex with Ca^{2+} which in turn binds to the enzymes, cyclic nucleotide phosphodiesterase and adenyl cyclase, with a resultant increase in enzyme activity (17-20, 27,28). Modulator protein is clearly an important regu-

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latory protein in animal tissues since it is involved in translating transmembrane Ca^{2+} fluxes into cellular responses. This protein has been found throughout the animal kingdom from coelenterates to mammals, and a recent observation indicates the presence of a modulator protein activity in higher plants (29).

The enzyme, NAD kinase, has been implicated as an important regulatory protein in higher plants since many key metabolic enzymes use NADP in preference to NAD. Illumination of plant leaves causes a conversion of NAD to NADP (30) and phytochrome has been implicated by Tezuka and Yamamoto (31) in the activation of NAD kinase. Recently, Muto and Miyachi (32) showed that NAD kinase in plants was activated by a protein activator which is heat stable and highly acidic. We noted that the activation of NAD kinase in plants and the properties of the plant activator protein are similar to modulator protein-dependent enzyme regulation in animal systems. The apparent difference is the dependence on Ca^{2+}. However, Muto and Miyachi (32) did not use metal chelators in their assays for NAD kinase and thus leave open the possibility of Ca^{2+}-dependent regulation. We report here the presence in plants of a Ca^{2+}-dependent, heat stable activator protein of NAD kinase that shows similarities to the modulator proteins isolated from animal sources.

MATERIALS AND METHODS. All reagents used were of the best commercial grade available. Luminescence measurements were determined as previously described (33-35). Bovine brain modulator protein was a gift from Dr. T. C. Vanaman and porcine brain modulator protein and modulator protein-dependent phosphodiesterase were gifts from Dr. F. L. Siegel. Renilla modulator protein was a gift from Drs. J. C. Matthews and H. P. Jones.

Plant proteins, NAD kinase and its activator, were obtained from seedlings of Pisum satavum L. cultivar willet wonder. Plants were germinated in trays of vermiculite and grown for 11 days in natural light at 25°. Two hundred grams of leaves were harvested into 600 ml of 25 mM Tris-HCl, pH 8.0 at 25°, containing 2% w/v polyvinylpolypyrrolidone. The tissue was homogenized for 3 min at 4° using a Willems Polytron PT-20st followed by filtration through cheesecloth and centrifugation at 12,000 g for 30 min. The supernatant was brought to 50% saturation with solid ammonium sulfate and allowed to equilibrate for 30 min. The resulting precipitate was removed by centrifugation and dialyzed to an electroconductivity equivalent to 0.1 M NaCl. The resultant solution was passed through a DEAE-cellulose column which had been pre-equilibrated with 2 mM Tris-HCl, pH 8.0, containing 0.1 mM EGTA. The NAD kinase did not bind to the DEAE-cellulose and was used without further purification. The activator was

Table I. ACTIVITY AND HEAT STABILITY OF PLANT ACTIVATOR PROTEIN

	Phosphodiesterase Activity	NAD-Kinase Activity
No activator	10	4
Unheated activator	50	29
Heated activator	45	24

The peak activity fraction from Figure 2 was used in these experiments. The plant activator protein preparation was heated to 85° for 1 min. The values for NAD-kinase activity relate to 0.01 absorbancy unit lost at 600 nm per 200 s.

removed from the DEAE-cellulose by a linear gradient, 0-0.6 M, of NaCl in 25 mM Tris-HCl, pH 8.0, containing 0.1 mM EGTA. The active fractions were pooled and heated to 85° for 1 min followed by centrifugation to remove the resultant precipitate. This supernatant was used without further purification as the activator of NAD kinase. The plant activator protein and animal modulator protein were run separately on a 2.8 X 143 cm Sephadex column, G-75 superfine, at 4° using a buffer consisting of 1.0 mM Tris-HCl, pH 7.8, containing 1.0 mM EDTA. The resulting fractions were assayed for their ability to stimulate phosphodiesterase.

The Ca^{2+}-dependent regulator proteins were assayed for their ability to stimulate the activity of modulator-dependent porcine brain phosphodiesterase using the bioluminescence assay of Matthews and Cormier (35). The NAD kinase was assayed by the method of Muto and Miyachi (32) except that the reaction buffer was as follows: 100 mM Tris-HCl, pH 8.0; 2 mM NAD; 3 mM ATP; 10 mM $MgCl_2$; 0.01 mM EGTA; 1.0 mM $CaCl_2$. For Ca^{2+} free assays the EGTA concentration was increased to 1.0 mM.

RESULTS AND DISCUSSION. Heated extracts of peas contain a protein which is capable of stimulating the activity of modulator protein-dependent porcine brain phosphodiesterase in a manner similar to that observed for modulator proteins isolated from animal sources (Table I.). As has been shown for animal modulator proteins (22-26) this stimulation is also Ca^{2+}-dependent (Fig. 1). Assuming that proteins in the crude mixture do not alter the calculated values for free Ca^{2+} in the Ca^{2+}-EGTA buffers, the pCa for 50% saturation is approximately 6.6. As shown in Table I, NAD kinase activity obtained from peas is also stimulated by these heated extracts and this stimulation is dependent on the presence of Ca^{2+}

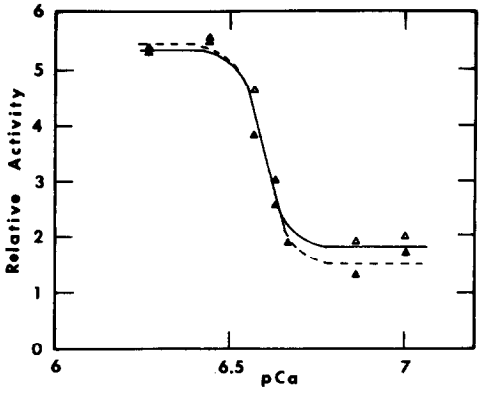

Figure 1. Calcium dependence of the plant activator protein-dependent stimulation of NAD-kinase (Δ-Δ) and modulator dependent-porcine brain phosphodiesterase (▲-▲). The assays were done in 25 mM Tris-HCl, pH 8.0 at 25°, 10 mM $MgCl_2$, 2.0 mM EGTA. The values for NAD-kinase activity relate to 0.01 absorbancy units lost at 600 nm per 200 s. Free Ca^{2+} in the Ca^{2+}-EGTA buffer system was determined using a modification of the program of Perrin and Sayce (40).

(Fig. 1). The Ca^{2+} concentration dependence of both activations is similar indicating that the K_d for Ca^{2+} is approximately the same for both processes. Heated extracts obtained from mungbean (Phaseolus aureus), spinach (Spinacia oleracea L.), and wild carrot (Daucus carota L.) also have the ability to stimulate modulator protein-dependent porcine brain phosphodiesterase. Conversely, modulator proteins isolated from porcine brain, bovine brain, and the coelenterate Renilla reniformis will also stimulate plant NAD kinase activity in the presence of Ca^{2+}. Table II shows the effect observed using bovine brain modulator protein.

Chromatography of plant activator protein for NAD kinase on DEAE-cellulose shows a single activity peak at a salt concentration of about 330 mM NaCl (Fig. 2). The phosphodiesterase stimulating activity co-elutes from the DEAE-cellulose column with this activator protein (Fig. 2) and both show the same stability when heated (Table I). The plant activator protein elutes from DEAE-cellulose in a manner similar to the NAD kinase activator protein described by Muto and Miyachi (32). The plant activator protein and animal modulator protein were found to have similar elution volumes on Sephadex G-75 superfine indicating similar Stokes' radii.

Table II. THE CA^{2+}-DEPENDENT STIMULATION OF PLANT NAD-KINASE ACTIVITY BY BOVINE BRAIN MODULATOR PROTEIN

Additions	Relative NAD-Kinase Activity
A. EGTA minus modulator protein	6.5
B. EGTA plus modulator protein	6.1
C. Ca^{2+} plus modulator protein	29
D. Ca^{2+} minus modulator protein	10.8*

*This NAD-kinase preparation was contaminated with small amounts of plant activator protein which accounts for the stimulation by Ca^{2+} in the absence of added modulator protein. The values relate to 0.01 absorbancy units lost at 600 nm per 200 sec.

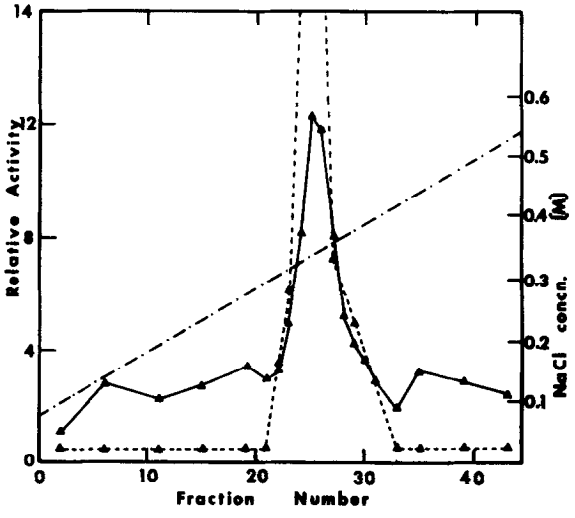

Figure 2. Chromatography of plant activator protein on DEAE-cellulose. Protein was eluted from the column by a linear, 0-0.6 M NaCl gradient. The electroconductivity of each fraction was measured and related to known concentrations of NaCl. The closest linear fit to the conductivity data was plotted (-·-). The activity of NAD-kinase plus 10 µl of each fraction was assayed (Δ-Δ), and the activity of modulator dependent-porcine brain phosphodiesterase plus 5 µl of each fraction was assayed (▲---▲). The peak tybes of phosphodiesterase stimulating activity saturated the assay at a 5-fold dilution. The values for NAD-kinase activity relate to 0.01 absorbancy unit lost at 600 nm per 200 s.

Using polyacrylamide gel electrophoresis, animal modulator proteins have been shown to replace troponin C in a Ca^{2+}-dependent complex with troponin I (36-37). In the absence of Ca^{2+}, the modulator protein moves to a position near the bottom of the gel and troponin I does not enter the gel. In the presence of Ca^{2+}, this same mixture shows a single band of complex near the top of the gel. Polyacrylamide gel electrophoresis of a mixture of crude plant activator plus troponin I (in the absence of Ca^{2+}) shows several bands with one in the region of animal modulator protein activity. In the presence of Ca^{2+}, this band is no longer visible. However, a new one appears near the top of the gel in a position occupied by animal modulator protein-troponin I complexes.

The data suggest that the plant activator activity is a protein with properties similar to the modulator proteins found in animals. This is supported by the cross reactivity of plant activator and modulator protein in the activations of mammalian brain cyclic nucleotide phosphodiesterase and plant NAD kinase. In addition, plant activator and modulator protein are similar in their dependence on Ca^{2+}, acidic natures, behavior on Sephadex, and their interactions with troponin I. The data implicates plant activator protein as a Ca^{2+}-dependent modulator of NAD kinase activity which suggests Ca^{2+} involvement in the regulation of plant metabolism by controlling the NADP-NAD ratio (30-32). These observations also suggest that Ca^{2+}-dependent regulatory proteins may constitute a regulator system which occurred in primitive eucaryotic cells and contemporary species, both plant and animal. Further, the recent finding that plants contain cyclic nucleotides (38) suggests even greater similarities between plant and animal intracellular regulation.

This is the first report that shows a possible link between a Ca^{2+}-dependent regulatory protein and a specific enzyme (i.e. NAD kinase) in higher plants. Calcium transients within animal cells have been linked to several intracellular control mechanisms and a number of these are modulated by a Ca^{2+}-dependent regulator protein as outlined in the Introduction. The possibility that NAD kinase

in plants is regulated by a Ca^{2+}-dependent regulator protein suggests that Ca^{2+} transients may be important in the regulation of plant processes as well. Calcium may therefore be involved as a second messenger in processes such as hormone activation and phytochrome control. The possibility that transmembrane Ca^{2+} transients are involved in the regulation of intracellular processes in plants is supported by the findings of Weisenseel and Ruppert (39) which reveal that Ca^{2+} is required for the phytochrome mediated depolarization of Nitella cells.

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