Analysis of the State of Posttranslational Calmodulin Methylation in Developing Pea Plants

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ABSTRACT

A specific calmodulin-N-methyltransferase was used in a radiometric assay to analyze the degree of methylation of lysine-115 in pea (Pisum sativum) plants. Calmodulin was isolated from dissected segments of developing roots of young etiolated and green pea plants and was tested for its ability to be methylated by incubation with the calmodulin methyltransferase in the presence of [3H]methyl-S-adenosylmethionine. By this approach, the presence of unmethylated calmodulins were demonstrated in pea tissues, and the levels of methylation varied depending on the developmental state of the tissue tested. Calmodulin methylation levels were lower in apical root segments of both etiolated and green plants, and in the young lateral roots compared with the mature, differentiated root tissues. The incorporation of methyl groups into these calmodulins samples appears to be specific for position 115 since site-directed mutants of calmodulin with substitutions at this position competitively inhibited methyl group incorporation. The present findings, combined with previous data showing differences in the ability of methylated and unmethylated calmodulins to activate pea NAD kinase (DM Roberts et al. [1986] J Biol Chem 261: 1491–1494) raise the possibility that posttranslational methylation of calmodulin could be another mechanism for regulating calmodulin activity.

Calmodulin is a highly conserved, ubiquitous, calcium-modulated protein that interacts with a number of enzymes and stimulates their activities (22). Nε-Trimethyllysine is a posttranslational modification that is found at position 115 of many calmodulins. This site is methylated by a S-adenosyl-L-methionine:lysine N-methyltransferase (15, 26). The question of the functional significance of calmodulin methylation is unclear. Although this modification is commonly found in calmodulin, naturally occurring calmodulins that have unmodified lysine at 115 rather than trimethyllysine have been purified and characterized from some organisms such as Chlamydomonas reinhardii (10), Dictyostelium discoideum (13), and trypanosomes (27). Additionally, active recombinant DNA-derived calmodulins that do not possess trimethyllysine 115 have been expressed in Escherichia coli from cloned calmodulin genes or cDNAs (16, 17, 20).

With respect to the regulatory activities of calmodulin, unmethylated calmodulins are not significantly different from methylated calmodulins in their abilities to bind calcium and activate a number of enzymes (16–18, 20, 26). However, in the case of one calmodulin-dependent enzyme, plant nicotinamide adenine dinucleotide kinase, the level of enzyme activation by unmethylated calmodulins is at least three-fold greater than the activation by methylated calmodulins (13, 19, 20). Further, this higher level of activation by unmethylated calmodulins can be reversed by enzymatic methylation

in vitro (21). The results raise the possibility that selective calmodulin activator activities could be attenuated by posttranslational methylation of lysine 115. For example, the methylation of calmodulin could result in an overall reduction in NAD kinase activation whereas other regulatory targets may not be similarly affected. These changes could be of significance to the regulation of nicotinamide coenzyme fluxes in the plant cell. The question that these observations raise is: do methylated and unmethylated derivatives of plant calmodulin exist in vivo?

By amino acid composition analysis (2, 3, 12, 33) and amino acid sequence determination (9, 28), it has been determined that calmodulins isolated from various higher plant tissues show the presence of one mole of trimethyllysine per mole calmodulin. These data would seem to suggest that the methylation of lysine 115 of higher plant calmodulin is stoichiometric. However, these calmodulins were purified from whole seedlings, and small or transient amounts of unmethylated or undermethylated calmodulins that are specifically localized in certain tissues may have evaded detection by protein chemistry methods. For example, studies with calmodulins from certain animal systems have shown that the levels of methylation can vary depending upon the tissues tested (14, 26). In the present work, we show the presence of pools of methylated and undermethylated calmodulins in developing pea plants by using a sensitive and specific radiometric assay based on the calmodulin methyltransferase enzyme.

MATERIALS AND METHODS

Materials

1H-AdoMet, 76.5 Ci/mmol was purchased from Du Pont. Nonradioactive AdoMet was obtained from Sigma. Sheep

Abbreviations: 1H-AdoMet, [3H-methyl]-S-adenosyl methionine; AdoMet, S-adenosyl-L-methionine; βME, 2-mercaptoethanol; PPVP, polyvinylpyrrolidone; RIA, radioimmunoassay; VU-1 calmodulin, calmodulin derived from a cloned synthetic gene; VU-3 calmodulin, VU-1 calmodulin with an arginine substitution at position 115; VU-4 calmodulin, VU-1 calmodulin with an isoleucine substitution at position 115.
brains were obtained from the Large Animal Clinic, School of Veterinary Medicine, University of Tennessee. Calmodulin-Sepharose 4B was prepared by the method of Klee and Krinks (7). All other reagents were purchased from commercial sources and were of the highest grade available.

Recombinant DNA derived VU-1, VU-3 (Lys to Arg-115), and VU-4 (Lys to Ile-115) calmodulins were produced by using the approaches described previously (20, 21, 23).

Preparation of Calmodulin-Lysine-N-Methyltransferase

Calmodulin methyltransferase was purified from sheep brains by using a modification of the procedures described for rat tissues (15, 26). One sheep brain (90 g) was homogenized in four volumes of 25 mM Hepes NaOH (pH 7.4), 4 mM βME, 0.25 mM sucrose, 5 mM EGTA, 0.1 mM PMSF, and was centrifuged for 30 min at 10,000 g. Supernatants were brought to 35% saturation with solid ammonium sulfate and were stirred for 30 min. The sample was centrifuged for 30 min at 12,000 g. Ammonium sulfate was added to the supernatant to a final concentration of 70% saturation and was stirred for 30 min. The sample was centrifuged for 30 min at 12,000 g. The pellet was resuspended in 30 mL of homogenization buffer (pH 6.8) without sucrose and then dialyzed overnight at 4°C against 4 L of this buffer with four changes. An equal volume of phosphocellulose equilibrium buffer (25 mM Hepes NaOH [pH 6.8], 4 mM βME, 0.1 mM PMSF, 0.01% (w/v) Triton X-100) was added to the dialyzed sample. The sample was applied to a 2.5 × 10 cm column of phosphocellulose, was washed with 400 mL of the equilibrium buffer, and was eluted with a linear NaCl gradient (0–0.5 M) in equilibrium buffer. The enzyme peak was pooled and concentrated by ultrafiltration in an Amicon stirred cell with a YM-10 membrane. The concentrated enzyme was dialyzed against calmodulin-Sepharose buffer, 5 mM Tris HCl (pH 7.4), 2 mM DTT, 0.1 mM NaCl, 0.1 mM CaCl₂, 0.01% (w/v) Triton X-100, 0.1 mM CaCl₂. The solution was applied to a 1.0 × 4 cm column of chicken gizzard calmodulin-Sepharose equilibrated in calmodulin-Sepharose buffer. The column was washed with the same buffer containing 0.5 mM NaCl. The enzyme was eluted with calmodulin-Sepharose elution buffer, 5 mM Tris HCl (pH 7.4), 2 mM DTT, containing 0.01% (w/v) Triton X-100, 1.0 mM NaCl, and 1 mM EGTA. The active fractions were pooled and concentrated by ultrafiltration and were applied to a 1.5 × 84 cm column of Biogel A-1.5 equilibrated in 25 mM Hepes NaOH (pH 7.4), 2 mM DTT, 0.15 mM NaCl, 0.01% (w/v) Triton X-100. The column was eluted at 4 mL/h with this buffer. Fractions of 1.4 mL were collected. The active fractions were pooled and applied to a 1.5 × 12 cm column of Sephadex G-10, previously equilibrated in 25 mM Hepes NaOH (pH 7.5), 2 mM DTT, 0.01% (w/v) Triton X-100. Active samples were pooled and were stored frozen at -80°C.

Analytical Methods

Radiometric calmodulin N-methyltransferase assay procedures were done as previously described (25). Assays were done under conditions where enzyme activity is constant with time and proportional to enzyme concentration. Typical reaction mixtures (total volume of 100 μL) contained VU-1 calmodulin (1.5 μg), 12 μM [3H]AdoMet (0.5 μCi), 0.1 mM NaCl, 2 mM MgCl₂, 5 mM DTT, 0.01% (w/v) Triton X-100, 0.1 mM CaCl₂, 0.1 mM glycyglycine NaOH (pH 8.0). Reactions were initiated by the addition of enzyme. The mixture was incubated for 20 min at 37°C and the reactions were terminated by heating at 90°C for 3 min. The samples were centrifuged in an Eppendorf model 3514 microfuge for 6 min. [3H]Calmodulin was separated from the other reaction components by calcium-dependent adsorption to phenyl-Sepharose. The supernatants were combined with 200 μL of phenyl-Sepharose equilibrated in 50 mM Tris HCl (pH 8.0), 0.3 mM NaCl, 0.1 mM CaCl₂, 0.1 mM EGTA, and was brought to a final concentration of 1:1 with solid ammonium sulfate and was stirred for 30 min. The sample was centrifuged for 30 min at 12,000 g. Ammonium sulfate was added to the supernatant to a final concentration of 70% saturation and was stirred for 30 min. The sample was centrifuged for 30 min at 12,000 g. The pellet was resuspended in 30 mL of homogenization buffer (pH 6.8) without sucrose and then dialyzed overnight at 4°C against 4 L of this buffer with four changes. An equal volume of phosphocellulose equilibrium buffer (25 mM Hepes NaOH [pH 6.8], 4 mM βME, 0.1 mM PMSF, 0.01% (w/v) Triton X-100) was added to the dialyzed sample. The sample was applied to a 2.5 × 10 cm column of phosphocellulose, was washed with 400 mL of the equilibrium buffer, and was eluted with a linear NaCl gradient (0–0.5 M) in equilibrium buffer. The enzyme peak was pooled and concentrated by ultrafiltration in an Amicon stirred cell with a YM-10 membrane. The concentrated enzyme was dialyzed against calmodulin-Sepharose buffer, 5 mM Tris HCl (pH 7.4), 2 mM DTT, 0.1 mM NaCl, 0.1 mM CaCl₂, 0.01% (w/v) Triton X-100, 0.1 mM CaCl₂. The solution was applied to a 1.0 × 4 cm column of chicken gizzard calmodulin-Sepharose equilibrated in calmodulin-Sepharose buffer. The column was washed with the same buffer containing 0.5 mM NaCl. The enzyme was eluted with calmodulin-Sepharose elution buffer, 5 mM Tris HCl (pH 7.4), 2 mM DTT, containing 0.01% (w/v) Triton X-100, 1.0 mM NaCl, and 1 mM EGTA. The active fractions were pooled and concentrated by ultrafiltration and were applied to a 1.5 × 84 cm column of Biogel A-1.5 equilibrated in 25 mM Hepes NaOH (pH 7.4), 2 mM DTT, 0.15 mM NaCl, 0.01% (w/v) Triton X-100. The column was eluted at 4 mL/h with this buffer. Fractions of 1.4 mL were collected. The active fractions were pooled and applied to a 1.5 × 12 cm column of Sephadex G-10, previously equilibrated in 25 mM Hepes NaOH (pH 7.5), 2 mM DTT, 0.01% (w/v) Triton X-100. Active samples were pooled and were stored frozen at -80°C.

Immunochromy

Antibodies against VU-1 calmodulin were produced in New Zealand White rabbits by subcutaneous injection of 1 mg VU-1 calmodulin emulsified 1:1 with Freund’s complete adjuvant. After 1 week the injection was repeated with 1 mg of VU-1 calmodulin in Freund’s incomplete adjuvant. After 2 additional weeks the injection was repeated again. The rabbit was bled at weekly intervals and the antisera was stored at -80°C.

Calmodulin concentrations were determined by competitive RIA by using the general method of Van Eldik and Lukas (32), with modifications described below. VU-1 calmodulin was iodinated by the chloramine T method as previously described (32). [125I]-VU-1 calmodulin (80,000 cpm) was incubated with anti-VU-1 calmodulin serum in a 200 μL reaction mixture containing 1.3% (v/v) normal rabbit sera, 2% (w/v) bovine serum albumin, 50 mM Tris HCl, 100 mM NaCl (pH 7.5), and various amounts of unlabeled calmodulin standards or samples. After 8 h at 4°C, 10 μL of goat-anti-rabbit IgG sera (Cappel Laboratories) was added and the reaction...
was continued at 30 °C for 1 h. An equal volume of 6% (w/v) PEG 8000 was added and the samples were placed on ice for 30 min. The precipitated antibody and antibody complexes were collected by centrifugation in an Eppendorf model 5814 microcentrifuge for 10 min. The pellets were washed once with 3% (w/v) PEG 8000 in 50 mM Tris HCl, 100 mM NaCl (pH 7.5), and were counted in a Beckman gamma 5500B counter. The amount of calmodulin in unknown samples was determined by constructing standard curves based on the ability of unlabeled calmodulin to compete with [125]I calmodulin.

Investigation of Endogenous Calmodulin Methylation in Pea Tissue

Pea seeds (Pisum sativum L. var Early Alaskan) were surface-sterilized with 5% (v/v) Clorox for 5 min and washed with water until the Clorox was removed. The seeds were aerated in water for 8 h and then germinated in trays of vermiculite in the dark at 22 °C to 24 °C. Etiolated plants were obtained by growing under these conditions for 72 h. Plants with roots 3.5 to 4.0 cm long were harvested, dissected into 0.6 cm root segments and epicotyls, and were analyzed as described below.

Green seedlings were germinated as described above, and were grown in a growth chamber at 22 °C to 24 °C for 6 d with 12 h light/dark cycles. Plants with primary roots of 9.0 to 9.5 cm were harvested, and were dissected into 1 cm root segments. Lateral roots, stems, and the young primary leaves also were dissected and analyzed as described below.

Pea calmodulin was extracted from the plant tissues by using a modification of the protocol described by Van Eldik et al. (31). This protocol takes advantage of the differential solubility of calmodulin in ethanol compared to most other cellular proteins. The procedure is rapid and yields calmodulin that is undegraded. Samples were frozen in liquid nitrogen and ground with a pestle in a mortar. The ground samples were thawed in a volume of homogenization buffer (75 mM Tris HCl [pH 7.5], containing 10 mM EDTA, 1% [w/v] PVPP, 2 mM thioglycolate, and 10 mM isocitrate) that was three times the wet weight of the tissue. The mixture was stirred and then centrifuged at 27,000 g at 4 °C for 20 min. The supernatant was brought to 50% (v/v) ethanol and stirred for 20 min. The sample was centrifuged for 20 min at 27,000 g. The pellet was discarded and ethanol was added to the supernatant to a final concentration of 80% (v/v) and stirred for 40 min. The sample was centrifuged for 30 min at 27,000 g at 4 °C. The pellet, which contains calmodulin, was lyophilized and resuspended with 50 mM Tris HCl (pH 7.5), 1 mM βME. The amount of calmodulin was quantitated by RIA.

To determine the degree of methylation, pea calmodulin samples were tested as substrates for the purified calmodulin N-methyltransferase from sheep brain. Pea calmodulin (1.5 μg) was incubated with 0.5 μg of purified enzyme and 12 μM [3H]AdoMet (1.5 μCi) at 37 °C for 4 h, in 50 μL of calmodulin N-methyltransferase assay mixture. For a positive control 1.5 μg of VU-1 calmodulin was treated under the same conditions. As negative controls VU-4 calmodulin and chicken gizzard calmodulin were incubated with enzyme. After 4 h, the reaction mixtures were heated at 90 °C for 3 min, then centrifuged for 6 min in a microfuge. 3H-Calmodulin was separated from other reaction components in the supernatant by phenyl-Sepharose chromatography and was counted by liquid scintillation spectrometry as described above. For fluorography, samples were subjected to SDS-PAGE after boiling for 5 min with SDS-PAGE sample buffer, and were fixed with 10% (v/v) acetic acid, 30% (v/v) methanol, and then incubated with EN3HANCE (Du Pont). The fluoros were precipitated with cold water and the gel was dried and exposed to Wicor-X RP x-ray film at −80 °C.

RESULTS

Purification of Calmodulin Methyltransferase and Design of Calmodulin Methylation Assay

To design a specific assay for the degree of methylation of plant calmodulins, it is necessary to use a highly purified, specific calmodulin methyltransferase. For this purpose the calmodulin methyltransferase from sheep brain was purified by the protocol summarized in Table 1. Overall, the purification protocol resulted in 28,000-fold purification and a specific activity of 12,000 pmol/min/mg. SDS-PAGE of the purified enzyme preparation revealed two bands: one major band with an apparent Mr of 38,000 and a minor band with an apparent Mr of 42,000. The native enzyme elutes from Biogel A-1.5 with an apparent mol wt of 38,000 (data not shown). Calmodulin was not detected in the final sample. Consistent with a previous report (15), the enzyme activity was stimulated by Ca2+ (Kd = 3 × 10−6) in the presence of physiological concentrations (5 mM) of Mg2+. This observation, along with the calcium-dependent nature of the inter-

Table 1. Purification of S-Adenosyl-L-Methionine:CaM-Lysine N-Methyltransferase from Sheep Brain

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Protein</th>
<th>Total Activity*</th>
<th>Specific Activity*</th>
<th>Purification</th>
<th>Recovery</th>
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<td>3,360</td>
<td>0.43</td>
<td>1</td>
<td>100</td>
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<tr>
<td>Ammonium sulfate</td>
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<td>2,330</td>
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<td>2.5</td>
<td>69.5</td>
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<tr>
<td>Phosphocellose</td>
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<td>12.3</td>
<td>28.5</td>
<td>73.5</td>
</tr>
<tr>
<td>Calmodulin-Sepharose</td>
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<td>1,340</td>
<td>8,600</td>
<td>20,000</td>
<td>40.0</td>
</tr>
<tr>
<td>Biogel A-1.5</td>
<td>0.037</td>
<td>477</td>
<td>12,100</td>
<td>28,100</td>
<td>13.3</td>
</tr>
</tbody>
</table>

* Expressed as pmol of [3H]-methyl groups incorporated into VU-1 calmodulin per min.  
  ** Standardized to mg of protein in each sample.
action of the enzyme with calmodulin-Sepharose, suggests that the enzyme prefers the calcium-bound form of calmodulin as a substrate.

Conditions for stoichiometric methylation of calmodulin were established as shown in Figure 1. VU-1 calmodulin, a recombinant DNA derived calmodulin that contains an unmethylated lysine at position 115, was selected as a substrate because of its high homology with plant calmodulins (20). The time course of VU-1 calmodulin methylation shows that stoichiometric methylation (3 mol of methyl groups/1 mol of calmodulin) was attained after 3 h (Fig. 1A).

The final product was heat-stable and SDS-gel electrophoresis and fluorography showed that the only detectable methylated product was calmodulin (Fig. 1B). In order to show specificity, chicken gizzard calmodulin and VU-4 calmodulin were used as negative controls. If methylation occurs specifically and irreversibly at position 115, these calmodulins should not serve as substrates since they contain trimethyllysine (chicken gizzard calmodulin) or isoleucine (VU-4 calmodulin) at position 115. In all cases, methylation was not detected by scintillation counting nor by fluorographic analysis (Fig. 1B). Overall, the results show that the purified calmodulin methyltransferase is highly specific for the 115 site and can stoichiometrically incorporate 3 mol of [3H]methyl groups into this site.

Detection of Undermethylated Calmodulin from Pea Tissue

To test for the presence of unmethylated calmodulins, the calmodulin pool isolated from various pea tissues was quantitated by RIA, and was tested for its ability to serve as a substrate for the calmodulin methyltransferase by using the conditions for stoichiometric methylation described above. Thus, if pea calmodulins are not completely methylated, they will be able to incorporate [3H]methyl groups from [3H]AdoMet.

The distribution of undermethylated calmodulins in 3-d-old etiolated pea plants is shown in Figure 2. In order to test different tissues at various states of development, roots were dissected into 0.6 cm segments and were analyzed. Undermethylated calmodulins were detected in all tissues tested, but the highest incorporation of [3H]methyl groups occurred with calmodulin isolated from the 0.6 cm tip portion of the root, and corresponds to a value of 0.5 mol methyl groups incorporated/mol calmodulin. The level declined greatly as calmodulin from successive segments were tested, and the level of [3H]methyl group incorporation into calmodulin isolated from the mature tissues at the base of the root was 12-fold lower than calmodulin from the root apex (Fig. 2A). SDS-PAGE and fluorography verified the pattern of incorporation, and showed that methylated calmodulin was the only product (Fig. 2B).

A similar pattern was also observed in 6-d-old green pea seedlings (Fig. 3). At this age, the tap root is much more developed, and small, young, 1.0 to 1.5 cm lateral roots are apparent at the base. The epicotyl consists of a very small stem and primary leaves. The plants were dissected as shown.

Figure 1. Stoichiometric methylation of VU-1 calmodulin. VU-1 calmodulin was incubated with purified calmodulin N-methyltransferase and [3H]AdoMet as described in “Materials and Methods.” A, Time course of [3H]methyl incorporation; B, fluorography of assay products. VU-1 calmodulin and negative controls (chicken gizzard calmodulin and VU-4 calmodulin) were incubated with calmodulin N-methyltransferase and then analyzed by SDS-PAGE on 15% polyacrylamide gels and fluorography. Each lane contains 0.17 μg of calmodulin. Lane 1, VU-1 calmodulin incubated with enzyme; lane 2, VU-1 calmodulin with no added enzyme; lane 3, VU-4 calmodulin incubated with enzyme; lane 4, chicken gizzard calmodulin incubated with enzyme. The arrow indicates the electrophoretic position of VU-1 calmodulin.
Calmodulin Methylation Inhibitors

The availability of site-specific mutants of calmodulin could provide interesting probes for future in vivo studies of the biological significance of calmodulin methylation in plants. VU-3 calmodulin (21) and VU-4 calmodulin (this study) are not substrates for the calmodulin N-methyltransferase. However, with exception of a substitution at position 115, these calmodulins have flanking sequences identical to VU-1 calmodulin, and they may be recognized by the methyltransferase enzyme. With this in mind, we investigated VU-3 and VU-4 calmodulins as inhibitors of calmodulin methylation. As shown in Figure 4, VU-3 calmodulin and VU-4 calmodulin show competitive inhibition of VU-1 calmodulin methylation with calculated inhibition constants (K_i) of 40 and 2.2 nM, respectively, compared with a K_i for VU-1 calmodulin of 50 nM (data not shown). Interestingly, VU-4 calmodulin, which contains isoleucine 115 instead of lysine 115, is a more potent inhibitor than VU-3 calmodulin which contains arginine 115. As an additional test of whether the methylation of plant calmodulins in Figures 2 and 3 was the result of specific incorporation into site 115, we tested the ability of added VU-4 calmodulin to inhibit pea calmodulin methylation (Fig. 5). Added VU-4 calmodulin inhibited the methylation of pea calmodulin in a dose-dependent manner.

in Figure 3 and were analyzed. The highest levels of methyl group incorporation were associated with calmodulin from the tip of the primary roots and from the young lateral roots (Fig. 3A). The values are somewhat lower than those found in etiolated root tips and correspond to about 0.26 to 0.28 mol/mol calmodulin. Calmodulins from most of the other root tissues appear to be nearly completely methylated since they were poor substrates for the enzyme. Undermethylated calmodulins were also readily observed in the young stem and primary leaves. Again, the liquid scintillation counting data are supported by fluorographic analysis (Fig. 3B). Overall, the data clearly show that small pools of undermethylated calmodulins exist in plant tissues, and that in developing roots the levels are highest in the youngest tissues.

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methylated lysine or a mixture of unmethylated and partially methylated residues (i.e., monomethylated and dimethylated lysines) remains to be determined.

Calmodulin isolated from apical roots or young lateral roots possessed a lower level of methylation of lysine 115 than calmodulin from more mature differentiated tissues. Apical root regions also appear to have higher overall levels of calmodulin compared with mature root tissues (1, 5, 29). Although calmodulin methylation levels were lower in the apical region of developing roots, it is still apparent that calmodulins from these tissues possess a substantial degree of methylation. In this regard, it is important to note that this region consists of several different tissues including the root cap, the apical meristematic region, and cells undergoing rapid cell expansion and differentiation. Future work on developing roots, as well as other developmental systems, will be of interest to determine whether unmethylated or undermethylated calmodulins are associated with one cell type or a particular developmental state.

It is possible that the undermethylated calmodulin present in young tissues represents newly synthesized calmodulin in proliferating cells, and is in the process of being methylated. Certainly, the distribution and levels of undermethylated calmodulins would seem to suggest that this pool is transient. However, it is interesting to note that undermethylated calmodulins are detected in elongating root tissues above the apex in etiolated plants, but not in green plants. Also, analysis of other plant samples (Oh and Roberts, unpublished data) have revealed the presence of undermethylated calmodulins in nonmeristematic tissues. Overall, the data suggest that undermethylated calmodulins are transient and prevalent in apical tissues undergoing cell proliferation, but are not exclusively localized in these tissues.

The present finding that undermethylated calmodulins exist in plants raises the question of the physiological relevance of calmodulin methylation. We have reported previously that

**DISCUSSION**

A calmodulin methyltransferase was purified from sheep brain and was used in a sensitive radiometric assay to evaluate the methylation state of pea calmodulins. By this approach we have shown that calmodulins isolated from pea tissues can serve as substrates for the methyltransferase, and that the level of [3H]methyl incorporation varied with the developmental state of the pea tissue. Several lines of evidence indicate that the [3H]-product results from the methylation of undermethylated pea calmodulins at lysine 115: (a) the reaction product is stable to heat and electrophoresis under alkali conditions and thus is characteristic of methylated lysine derivatives (26); (b) all methylated products bind to phenyl-Sepharose in a calcium-dependent manner and comigrate with plant calmodulin on SDS-gels; (c) the enzyme used shows high specificity for calmodulins with lysine-115; and (d) methylation of pea calmodulins is inhibited in a competitive manner by site-specific mutants with substitutions at position 115. Whether these undermethylated calmodulins consist primarily of un-

**Figure 4.** Inhibition kinetics of the calmodulin N-methyltransferase. Inhibition by VU-3 and VU-4 calmodulins with VU-1 calmodulin as the variable substrate at constant concentrations of 12 µM S-adenosyl methionine. A. The concentrations of VU-3 CaM were 200 nM (●), 100 nM (▲), 50 nM (◆), 0 nM (□). B. The concentrations of VU-4 CaM were 12 nM (●), 6 nM (▲), 2 nM (◆), 0 nM (□).

**Figure 5.** Inhibition of methylation of pea calmodulin by VU-4 calmodulin. Calmodulin (1.25 µg) from pea was treated with calmodulin N-methyltransferase and 6.0 µM [3H]AdoMet (1.5 µCi) in the absence or in the presence of VU-4 calmodulin. The results are expressed as % of control (no added VU-4 calmodulin).
calmodulins with trimethyllysine 115 activate pea NAD kinase to a level that was at least threefold lower than the level of activation obtained with unmethylated calmodulins (13, 19-21). The results suggest that the level of the NAD kinase activation by calcium and calmodulin within a particular cell could be affected by the degree of calmodulin methylation. Whether NAD kinase activity is affected by alterations in the calmodulin methylation state in vivo remains to be determined. However, it is clear that the distribution of undermethylated calmodulin derivatives may not necessarily parallel the level of calcium-calmodulin stimulated NAD kinase in the plant. For example, Allan and Trewavas (1) detected low levels of calcium-stimulated NAD kinase activity in extracts of the apical portion of etiolated pea roots, and showed that activity increased significantly between 0.5 to 3 cm above the apex, in the zones of rapidly elongating root cells and mature root tissue. Further analyses of the distribution of calmodulin, calmodulin methyltransferases, NAD kinase protein levels, and cytosolic NAD/NADP ratios in other plant tissues may provide more insight into whether endogenous calmodulin methylation affects this regulatory system.

In addition, our results suggest that calmodulin mutants may be useful in analyzing the biological relevance of calmodulin methylation in plants. Site-directed mutants of calmodulins with substitutions at position 115 showed competitive inhibition of the calmodulin methylation. Thus, the results suggest that the recognition site for the enzyme is still intact in these derivatives. Previous work with VU-3 calmodulin showed that it is active and retains the higher maximal NAD kinase activator properties of the unmethylated calmodulins but was no longer susceptible to the effects of the methyltransferase (21). Hence, site-directed mutants of calmodulin with substitutions at position 115 appear to retain calmodulin activity. This is supported by studies of Schizosaccharomyces pombe calmodulin which contains an arginine instead of a lysine at position 115 (30). From the perspective of plants, the introduction of foreign calmodulin derivatives with substitutions at position 115 may inhibit endogenous calmodulin methylation while retaining high NAD kinase stimulatory activity in vivo. Further, VU-4 calmodulin (Lys to Ile-115) showed 20 times higher inhibitor activity than VU-3 calmodulin (Lys to Arg-115). Thus, it may be possible to design even more effective inhibitors of calmodulin methylation.

An important question for future studies is: what factors control calmodulin methylation in plants? The finding of developmental differences in methylation state raises the possibility that this is another facet of the calmodulin regulatory system. Calmodulin methylation is stimulated by micromolar calcium concentrations. A possible explanation for this is that the altered conformation of calmodulin that results from calcium binding may be preferred by the methyltransferase. Thus, calcium ion fluxes may play a role in controlling the rate and degree of calmodulin methylation. In this regard it is of interest that a certain motility mutant of Paramecium possesses a calmodulin mutant with a substitution of a threonine for a valine residue at position 136 in the fourth calcium-binding site, and shows a reduced level of methylation at position 115 (11).

Another potential step for the control of calmodulin methylation could be at the level of calmodulin turnover. For example, based on in vitro studies with unmethylated and methylated calmodulins, it has been proposed that lysine 115 of calmodulin is a site for ubiquitin conjugation, and that methylation may protect calmodulin from degradation by the ubiquitin-dependent proteolytic pathway (6). It remains undetermined whether unmethylated calmodulins are conjugated to ubiquitin in vivo and are turned over more rapidly than their methylated counterparts.

The degree of calmodulin methylation also could be controlled by the levels of the calmodulin methyltransferase present in the cell. Rowe et al. (26) noted that tissues with undermethylated calmodulins possess significantly lower levels of the calmodulin methyltransferase activity. Presently, little is known regarding the properties of plant calmodulin methyltransferases. Future studies with these enzymes should provide further insight into the control of calmodulin methylation and its importance in cell regulation.

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LITERATURE CITED


