Analysis of Suborganellar Fractions from Spinach and Pea Chloroplasts for Calmodulin-binding Proteins

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ABSTRACT Purified chloroplasts from spinach and pea leaves were subfractionated into envelope, thylakoid, and stroma fractions and were analyzed for calmodulin-binding proteins using a ^{125}I-calmodulin gel overlay assay. Calmodulin binding was primarily associated with a major polypeptide (M_r 33,000) in the envelope membrane fraction. In contrast, major calmodulin-binding proteins were not detected in the thylakoid or stroma fractions. Our results provide the first evidence of calmodulin-binding proteins in the chloroplast envelope, and raise the possibility that calmodulin may contribute to the regulation of chloroplast function through its interaction with calmodulin-binding proteins in the chloroplast envelope. In addition, our results combined with those of other investigators support the proposal that subcellular organelles may be a primary site of calmodulin action.

Calcium is required for a number of cellular activities in eucaryotes, and has been implicated as a possible regulatory agent of metabolic activities and growth responses in a variety of plant species (1). Kinetic, thermodynamic, and structural data strongly suggest that the major intracellular receptors involved in calcium-mediated regulation are a class of calcium-binding proteins known as calcium-modulated proteins (for a recent review, see reference 2). Calmodulin is a calcium-modulated protein that has been found in all eucaryotes examined, including higher plants (for reviews, see references 1–3), and appears to be one of the most highly conserved proteins known (1, 4).

Calmodulin has a number of in vitro biochemical activities including the ability to stimulate certain enzyme activities and bind to proteins (1–3). Two calmodulin-sensitive enzyme activities have been demonstrated in higher plants: a NAD kinase (5, 6) and a microsomal calcium ATPase (7). A thorough investigation of the properties of calmodulin-sensitive enzymes and calmodulin-binding proteins is necessary before the role of calmodulin in the regulation of cellular processes in plants can be completely understood. An important part of such an investigation is the determination of the subcellular distribution of calmodulin, calmodulin-stimulated enzymes, and calmodulin-binding proteins. The results of a number of subcellular fractionation and immunocytology studies carried out on tissues from vertebrate organisms indicate that, although calmodulin is found predominantly in the freely soluble fraction, small quantities appear to be associated with subcellular organelles (2, 8). In addition, calmodulin-binding proteins have been demonstrated in isolated organelles including mitochondria from 3T3 cells (9), and nuclear, mitochondrial/lysosomal, and microsomal fractions from chicken embryo fibroblasts (8).

In contrast to the studies on animal systems, information on the subcellular distribution of calmodulin and calmodulin-binding proteins in plant tissues is more limited. Recently, Jarret et al. (10) and Muto (11) have reported the presence of small quantities of calmodulin in the chloroplast fractions of pea and wheat, respectively. Jarrett et al. (10) suggested that this small pool of calmodulin is involved in the light-dependent phosphorylation of NAD by chloroplast NAD kinase. However, Simon et al. (6) demonstrated soluble and chloroplast pools of NAD kinase activity in spinach and found that the soluble enzyme was stimulated by calmodulin in the presence of calcium, whereas the chloroplast enzyme was not. Simon et al. (6) also found small quantities of calmodulin activity in spinach chloroplasts, but suggested that this chloroplast calmodulin may be the result of contamination by cytoplasmic calmodulin. A detailed analysis of chloroplast fractions and subfractions for calmodulin-binding proteins may provide additional insight into the physiological significance of the chloroplast calmodulin pool. In the present study, we examined purified chloroplast subfractions from spinach and pea leaves for calmodulin-binding proteins.

MATERIALS AND METHODS

Spinach leaves were obtained from a local vendor. Pea seeds (*Pisum sativum* var. Early Alaska) were surface sterilized with a 10% (vol/vol) solution of

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Chlorox, imbibed overnight, and grown in soil (Terra Lite Metromix 200, W. R. Grace and Co., Baltimore, MD) in a greenhouse under normal day-light. Leaves from 2–3-wk-old seedlings were used for chloroplast preparation.

Purified intact chloroplasts from spinach or pea leaves were prepared as described by Cline et al. (12), using a modification of the silica sol-density gradient method of Morgenthaler et al. (13). Plastids collected from Percoll gradients were washed twice in 5–10 vol of 50 mM HEPES/NaOH, pH 7.5, containing 0.33 M sorbitol. The chloroplasts were lysed by resuspension in 50 mM HEPES/NaOH, pH 7.5, containing 4 mM $MgCl_2$. Subfractionation of the lysed chloroplasts was achieved using a modification of the discontinuous sucrose gradient centrifugation method of Mendiola-Morgenthaler and Morgenthaler (14). The lysed chloroplast suspension (6 ml, 0.2 mg/ml chlorophyll) was layered on a step gradient consisting of 2 ml of 30% (wt/vol) sucrose and 2.5 ml of 12% (wt/vol) sucrose, and was centrifuged at 21,500 rpm (Beckman SW 41 rotor; Beckman Instruments, Inc., Palo Alto, CA) for 1 h at 4°C. The stroma fraction remains at the top of the gradient and the thylakoid forms a pellet at the bottom. The envelope fraction, which bands at the 30%/12% sucrose interface, was diluted in 50 mM HEPES/NaOH, pH 7.5, and was collected by sedimentation at 35,000 rpm (SW 41 rotor) for 1 h. All fractions were stored at −80°C until analysis.

Calmodulin was purified (15) and radiolabeled with ^{125}I using the lactoperoxidase procedure (16) as previously described. Samples were subjected to electrophoresis on polyacrylamide gels using the buffer system of Laemmli (17) and a modified microslab apparatus similar to that described by Matsudaira and Burgess (18). Detection of calmodulin-binding proteins was accomplished using the ^{125}I-calmodulin gel binding assay described by Van Eldik and Burgess (8), except that 0.1 mM $CaCl_2$ was used for the calcium incubations. Crude fractions from chicken gizzard that contain known calmodulin-binding proteins were included in each overlay assay as a positive control. Competition experiments were performed by incubating the gels with unlabeled calmodulin or α-lactalbumin for 30 min prior to the addition of ^{125}I-calmodulin.

Protein concentrations were determined by a modification of the procedure of Lowry et al. (19), as described by Markwell et al. (20) or by amino acid analysis. Chlorophyll was determined by the method of Arnon (21).

RESULTS AND DISCUSSION

Chloroplasts were purified by silica sol density gradient centrifugation (13) and were lysed and subfractionated using carefully defined conditions (14) to ensure fraction purity. The polypeptide profiles obtained by SDS gel electrophoresis of the chloroplast subfractions (Fig. 1*A*) appear comparable to those reported by other investigators (12, 22, 23).

Gel overlay analysis of the chloroplast subfractions from spinach and pea indicates the presence of a major calmodulin-binding protein in the envelope membrane (Fig. 1, *B* and *C*). This calmodulin-binding activity is associated with the major Coomassie-Blue-staining polypeptide (apparent M_r 33,000) in our envelope preparation (Fig. 1*A*). In addition to this major binding protein, the envelope fraction contains three minor calmodulin-binding proteins (apparent M_r 17,000, 27,000, and 50,000). A minor calmodulin-binding protein (apparent M_r 20,000) was also found in the pea thylakoid fraction. Calmodulin-binding proteins were not detected in the thylakoid fraction from spinach chloroplasts or in the stroma fractions from pea or spinach chloroplasts. Although the data shown in Fig. 1 represent the results of one experiment, the results obtained with four separate experiments on four different chloroplast subfraction preparations indicate the M_r 33,000 envelope polypeptide is the major calmodulin-binding protein. We were not able to detect the minor calmodulin-binding proteins in all envelope preparations (Fig. 2*A*).

The association of ^{125}I-calmodulin with the major envelope calmodulin-binding protein (M_r 33,000) appears to be calcium independent since binding occurs regardless of whether calcium or EDTA is present (Fig. 1, *B* and *C*). The calcium-independent interaction of calmodulin with calmodulin-binding proteins in gel overlay analysis is not unique to the calmodulin-binding proteins in the chloroplast envelope. Van Eldik and Burgess (8) found a number of calcium-independent calmodulin-binding proteins in the particulate subcellular fractions from chicken embryo fibroblasts. Our results combined with those of Van Eldik and Burgess (8) suggest that the calcium-independent interaction of calmodulin with particulate calmodulin-binding proteins may be a general phenomenon in plant and animal systems. It is important to recognize that a calcium-independent interaction does not preclude a calcium-dependent regulation of activity.

The binding of ^{125}I-calmodulin to the major envelope mem-

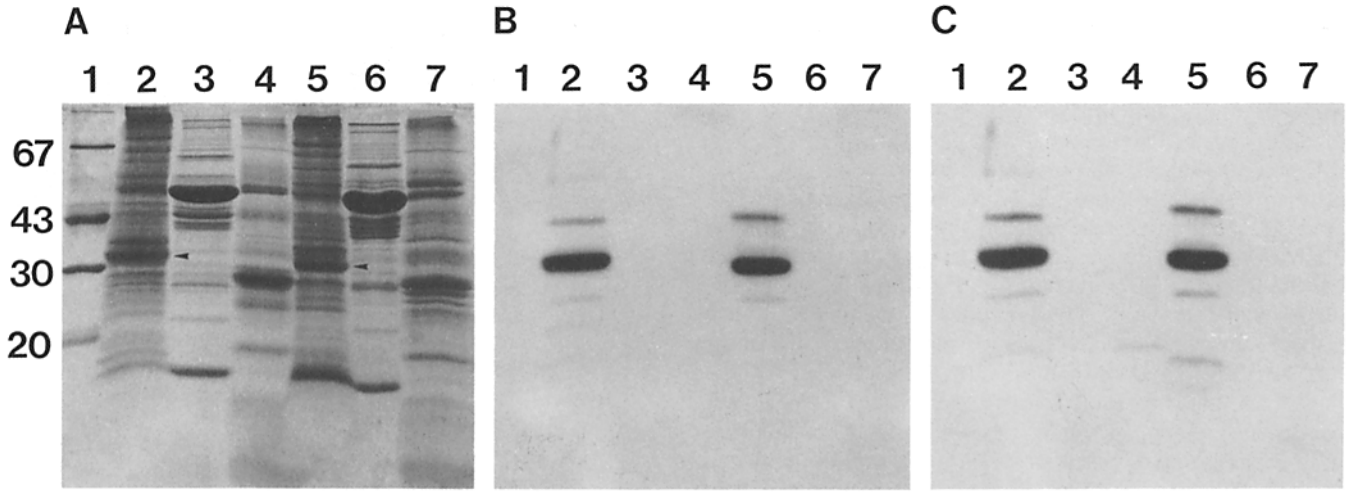

FIGURE 1 ^{125}I-Calmodulin gel overlay analysis of chloroplast fractions from spinach and pea. *A* shows a Coomassie-Blue-stained 15% (wt/vol) acrylamide gel and *B* and C show autoradiograms (10-h exposure). Gel overlay analysis was done in the presence of 0.1 mM $CaCl_2$ (*B*) or 5 mM EDTA (C) as described in Materials and Methods. Lane *1*: molecular weight standards: bovine serum albumin, M_r 67,000; ovalbumin, M_r 43,000; carbonic anhydrase, M_r 30,000; and soybean trypsin inhibitor, M_r 20,000). Lane *2*: chloroplast envelope fraction from pea. Lane *3*: stroma fraction from pea. Lane *4*, thylakoid fraction from pea. Lane *5*: envelope fraction from spinach. Lane *6*: stroma fraction from spinach. Lane *7*: thylakoid fraction from spinach. The amount loaded onto the gels accounts for ~0.15%, 0.15%, and 10% of the stroma, thylakoid, and envelope fractions, respectively. The arrowheads in *A* indicate the polypeptides which correspond to the major calmodulin-binding polypeptides (apparent M_r 33,000) in *B* and *C*. The Coomassie-Blue-stained gel in *A* corresponds to the autoradiogram in B. Nearly identical polypeptide profiles were obtained for the gel corresponding to the autoradiogram in C (data not shown).

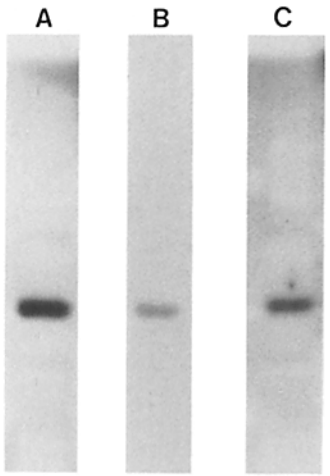

FIGURE 2 Inhibition of ^{125}I-calmodulin binding to the major calmodulin-binding protein (M_r 33,000) in spinach chloroplast envelopes. *A*, *B*, and *C* show autoradiograms (10-h exposure). The Coomassie-Blue-stained patterns were indistinguishable from those shown in Fig. 1 *A*. Gel overlay analysis was performed in the presence of 0.1 mM $CaCl_2$ and in the absence of competing protein (*A*), or in the presence of a 1,000-fold molar excess of unlabeled chicken gizzard calmodulin (*B*) or a 1,000-fold molar excess of unlabeled bovine α-lactalbumin (*C*). The gels in each panel contain equal quantities of an envelope membrane fraction from spinach chloroplasts.

brane polypeptide (M_r 33,000) is inhibited by pre-incubation with a 1,000-fold molar excess of unlabeled chicken gizzard calmodulin (Fig. 2*B*). In contrast, the binding of ^{125}I-calmodulin is only weakly inhibited, if at all, by a 1,000-fold molar excess of α-lactalbumin (Fig. 2*C*), a small, acidic, calcium-binding protein that has no known structural or functional relationship to calmodulin or other calcium-modulated proteins. These observations indicate that the interaction of ^{125}I-calmodulin with the major envelope binding protein is not an artifact of the labeling protocol, and that this binding protein is selective in its interaction with acidic calcium-binding proteins. Although these observations suggest that the interaction of calmodulin with the envelope binding proteins is relatively specific, it must be recognized that calmodulin may not be the endogenous receptor for these binding proteins. For example, calmodulin can substitute for troponin C under in vitro conditions as the calcium-sensitizing component of muscle actomyosin ATPase (24, 25). Therefore, on the basis of precedents with animal systems, it is possible to conjecture that a calcium-modulated protein similar to calmodulin may be the physiologically relevant protein.

Our results suggest that the chloroplast envelope may be a primary target of calcium regulation in the chloroplast. The envelope membrane is an integral component of the chloroplast, and participates in several chloroplast processes including the transport of metabolites (26), the uptake and incorporation of cytoplasmically synthesized proteins (27), and a number of metabolic activities, such as fatty acid metabolism (28, 29), galactolipid biosynthesis (30), prenylquinone biosynthesis (31), and carotenoid biosynthesis (32, 33). The calcium dependence of these activities has not been investigated. Simon et al. (6) investigated thylakoid, envelope, and stroma fractions from spinach chloroplasts for calmodulin-stimulated NAD kinase activity. Nearly all chloroplast NAD kinase activity was present in the stroma fraction and was not stimulated by calmodulin.

Data regarding the structure and function of the polypeptides of the chloroplast envelope are limited. Of particular relevance to the present work are the results of Flugge and Heldt (34–36) which suggest that a phosphate translocator activity is associated with the major polypeptide (M_r 29,000) in their envelope preparation. Whether the polypeptide of Flugge and Heldt corresponds to the major calmodulin-binding protein (M_r 33,000) in our envelope preparation remains to be determined.

The demonstration of calmodulin-binding proteins in the chloroplast envelope is an important step towards identifying the site of calmodulin regulation of chloroplast function. Clearly, more detailed analyses of the interactions between calmodulin and envelope proteins are necessary before any physiological significance can be attributed to these observations. However, as more becomes known regarding the structure and function of the envelope polypeptides, and the effect of calmodulin and calcium on their activities, a more defined role for calmodulin in the regulation of chloroplast processes may emerge.

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