Distribution of calmodulin within wheat leaf cells

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Subcellular distribution of calmodulin within wheat leaf cells was measured by a enzymatic method (activation of calmodulin-deficient NAD kinase from pea seedlings) or a radioimmunoassay. Most of cellular calmodulin was localized in cytosol fraction (89–93%), and remainders were in the mitochondrial (5–9%), the chloroplast (1–2%) and the microsomal (< 1%) fractions. Calmodulin concentration in the chloroplast was sufficient to saturate NAD kinase which was localized mostly in the chloroplast. Therefore, the light-induced Ca^{2+} transport in intact wheat chloroplasts [Muto,S. et al. FEBS Lett. (1982) 139, 250–254] may play an important role in regulation of chloroplast NAD kinase activity.

Calmodulin *Enzymatic assay* *Radioimmunoassay* *NAD kinase regulation* *Wheat leaf cell*

1. INTRODUCTION

The heat-stable, acidic and multifunctional Ca^{2+}-binding protein, calmodulin, modulates at least 2 enzymes in plants; i.e., NAD kinase [1–3] and Ca^{2+}-ATPase [4]. NAD kinase has been isolated from pea seedlings and shown to be absolutely dependent on calmodulin [1,5]. Plant NAD kinase plays an important role in the photo-regulation of nicotinamide coenzyme levels. Conversion of NAD to NADP occurs in algal cells such as *Chlorella* [6,7] and *Chlamydomonas* [8], and higher plant leaves [8,9] when they are illuminated. In leaves, this conversion occurs in the chloroplasts where most of NAD kinase is localized [9]. We have found that illuminated intact wheat chloroplasts actively took up Ca^{2+} [10]. These facts suggest that calmodulin is present in the chloroplasts and is activated when Ca^{2+} levels are elevated upon illumination. The subcellular distribution of calmodulin has been reported with animal cells [11–13]. According to C.C. Black, ~1% of total calmodulin localized in the chloroplast of pea leaves (personal communication). We report here that calmodulin is localized in at least 2 plant organelles, chloroplast and mitochondrion. The percent distribution in the chloroplast is very small. However, the concentration in the stroma is sufficient to saturate NAD kinase.

2. MATERIALS AND METHODS

Wheat (*Triticum aestivum* L.) was grown, mesophyll cell protoplasts were isolated and purified as in [9]. Purified protoplasts were suspended in 20 mM Hepes–KOH buffer (pH 7.6) containing 0.4 M sorbitol, 1 mM EDTA and 0.2% (w/v) bovine serum albumin and ruptured by passing through nylon mesh (opening, 20 μm), then centrifuged at 1500 × *g* for 2 min. The precipitated chloroplasts were suspended in the above medium and recentrifuged as above. The supernatants were combined and centrifuged at 20 000 × *g* for 30 min to precipitate mitochondria. The pellet was resuspended in the same medium and recentrifuged. The 20 000 × *g* supernatants were combined and centrifuged at 100 000 × *g* for 1 h to precipitate microsomes. The resulting pellet was resuspended and recentrifuged as above. The 100 000 × *g* supernatants were combined and used as cytosol fraction. Pellets of chloroplasts, mitochondria and microsomes were finally suspended in 20 mM Hepes–KOH buffer (pH 7.6) containing 1 mM EDTA. The chloroplasts were osmotically broken as above, and the mitochondria were sonically broken (20 kHz for 1 min). Both fractions were then centrifuged at 100 000 × *g* for 1 h. Precipitates and supernatants were used as the thylakoid and the stroma for chloroplast fraction

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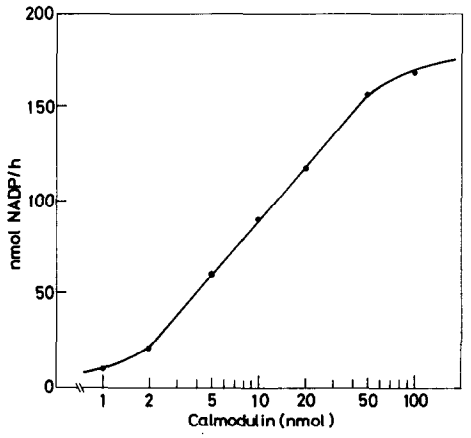

Fig.1. Calibration curve of spinach calmodulin for the enzymatic assay.

and the membrane and the matrix for mitochondrial fraction, respectively. All the above operations were carried out at 0–4°C. The subcellular fractions were then heated for 2 min in a boiling water bath. After cooling they were centrifuged at 20 000 × *g* for 30 min to remove coagulated proteins. Clear supernatants were used for calmodulin assay.

Calmodulin was assayed essentially as in [1]. The reaction mixture contained in 0.5 ml, 100 mM Tricine–KOH buffer (pH 8.0), 3 mM NAD, 3 mM ATP, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.2 units (when fully activated) of NAD kinase and appropriate amount of sample (calmodulin). The reaction was started by adding the enzyme and terminated by adding 100 μl 1 N HCl after 10 min at 37°C. The reaction product, NADP was assayed as in [9]. Calmodulin was also assayed by a radioimmunoassay (RIA) using calmodulin ^{125}I RIA kit (New England Nuclear) according to the manufacturer's instructions. For both assay methods, calibration curves were made using purified spinach calmodulin. Fig.1 and 2 are calibration curves for the enzymatic assay and RIA, respectively. The linear portions of the curves were used for calibration. Both methods showed similar sensitivity for spinach calmodulin. In the RIA, animal calmodulin was 10-times sensitive than plant calmodulin.

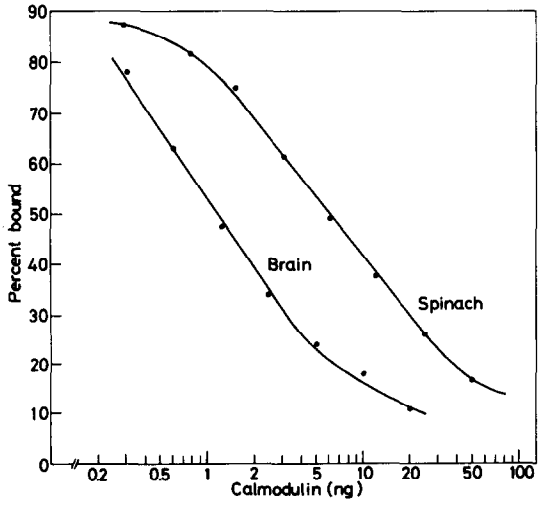

Fig.2. Calibration curve of spinach and bovine brain calmodulin for the radioimmunoassay.

Spinach calmodulin was purified to homogeneity essentially as in [14]. Calmodulin-deficient NAD kinase was prepared as in [1], and passed through a Sephadex G-25 column pre-equilibrated with 20 mM Hepes–KOH buffer (pH 7.6). Concentration of purified spinach calmodulin was determined by $\varepsilon_{276}^{1\%}$ of 0.9 [3]. Chlorophyll was assayed as in [15].

3. RESULTS AND DISCUSSION

Table 1 shows that distribution of NAD kinase was similar to that of NADPH-dependent glyceraldehyde 3-phosphate dehydrogenase, a marker enzyme for the chloroplast stroma. This indicates that NAD kinase is a stromal enzyme. Calmodulin was localized mostly in the cytosol fraction (88%), and the remainders were in the mitochondrial (7.8%), the chloroplast (3.6%) and the microsomal (0.6%) fractions. Since the distribution in each organelle fractions were < 10%, it is possible that this represents non-specific binding of cytosolic calmodulin to these organelles. To test this possibility, ^{125}I-labeled calmodulin (New England Nuclear) was added to the ruptured protoplasts and the organelles were fractionated. Radioactivities recovered in the chloroplast and the mitochondrial fractions were 2.6 and 0.6%, respectively (table 2). These may represent non-specific binding of cytosolic calmodulin to these organelles. In a parallel experiment, the endogenous calmodulin was assayed by both the enzymatic method and the RIA. The values thus obtained

Table 1

Distribution of calmodulin, NAD kinase and NADPH-dependent glyceraldehyde 3-phosphate dehydrogenase within wheat mesophyll cells[a]

Subcellular fraction	Calmodulin (μg)		NAD kinase (nmol/h)		Glyceraldehyde 3-phosphate dehydrogenase (μmol/min)	
Cytosol	20.79	(88.0%)	224	(4.4%)	1.90	(5.7%)
Microsome	0.14	(0.6%)	20	(0.4%)	0.02	(0.1%)
Mitochondria	1.84	(7.8%)	119	(2.3%)	0.28	(0.8%)
matrix	1.68	(7.1%)	88	(1.7%)	0.24	(0.7%)
membrane	0.16	(0.7%)	31	(0.6%)	0.04	(0.1%)
Chloroplast	0.85	(3.6%)	4701	(92.8%)	31.39	(93.5%)
stroma	0.66	(2.8%)	4403	(86.9%)	25.12	(74.8%)
thylakoid	0.19	(0.8%)	298	(5.9%)	6.27	(18.7%)

[a] Protoplasts used were equivalent to 1.96 mg chl

were corrected for non-specific binding assuming that the chloroplast and the mitochondrial fractions were contaminated with 2.6% and 0.6% of total calmodulin, respectively. Calmodulin contents in the chloroplast and the mitochondrial fractions determined by the enzymatic method were 0.27 μg and 1.17 μg, respectively. The respective values after the RIA were 0.08 μg and 0.32 μg. Applying the same correction factors to table 1, calmodulin contents in the chloroplast and the mitochondrial fractions were calculated to be 0.24 μg and 1.70 μg, respectively. In table 2, the values obtained by the RIA were smaller than those obtained by the enzymatic method. If NAD kinase is activated by activator (s) other than calmodulin in the sample, the enzymatic method would give higher values than the RIA. To test this possibility, either 0.5 mM chlorpromazine or 1 mM EGTA was added to the assay mixture. The enzyme was completely inactivated by either of them when activated by the cytosol, the chloroplast or the mitochondrial fractions. Calmodulin activities of these fractions were absorbed on and desorbed from a fluphenazine-conjugated Afi-Gel 10 column as a Ca^{2+}-dependent manner. No activity was detected with the pass through fractions (not shown). These facts indicate that the values obtained by the enzymatic method is due only to calmodulin. If sub-

Table 2

Distribution of calmodulin and endogenous ^{125}I-labeled calmodulin within wheat mesophyll cells[a]

Subcellular fraction	Calmodulin (μg)				^{125}I-Calmodulin (cpm)	
	Radioimmuno-assay		Enzymatic assay			
Cytosol	3.10	(86.1%)	19.00	(90.0%)	106.110	(96.8%)
Chloroplast	0.17	(4.7%)	0.82	(3.9%)	2843	(2.6%)
Mitochondria	0.34	(9.4%)	1.30	(6.2%)	659	(0.6%)

[a] Protoplasts used were equivalent to 2.45 mg chl

cellular fractions contained substance (s) which inhibits binding of calmodulin to the antibody, the RIA would give underestimations. Addition of subcellular fractions to the RIA system for the standard spinach calmodulin had no effect. Therefore the existence of the interference substance for the RIA would have been excluded. The reason for the inconsistence between these two assays is not known. However, these results clearly show that calmodulin is located in the chloroplasts and mitochondria.

The concentration of calmodulin in the chloroplast is especially important with respect to the regulation of NAD kinase. Chlorophyll contents in the chloroplast fraction were 1.96 mg and 2.45 mg in table 1 and 2, respectively. Assuming the stromal space of 30 μl/mg chl [16] and all chloroplast calmodulin are present in the stroma, the stromal concentration of calmodulin are calculated to be 4.08 μg/ml in table 1 and 1.09 and 3.68 μg/ml in table 2. The half-saturation concentration for the pea NAD kinase was 22 ng/ml (see fig.1). Therefore the enzyme would be clearly saturated with calmodulin in the stroma. The activity of NAD kinase in wheat chloroplast is sufficient to account for the light-induced conversion of NAD to NADP [9]. These facts suggest that the change in $[Ca^{2+}]$ in the stroma plays an important role in the regulation of NAD kinase. The light-driven Ca^{2+} transport in intact wheat chloroplast has relatively high K_m (180 μM), however has a very high maximum rate (30 $\mu mol \cdot h^{-1} \cdot mg\ chl^{-1}$) [10]. The cytoplasmic concentration of free Ca^{2+} in plants is suggested to be within 10^{-7}–10^{-5} M [17]. Even if the cytoplasmic concentration is lower than the K_m for Ca^{2+} transport, it may be possible to cause the accumulation of Ca^{2+} in the chloroplast, since a inflow of only 1 nmol Ca^{2+}/mg chl could raise stromal Ca^{2+} from 0–30 μM.

The mitochondria contained 3–7% of the total cellular calmodulin, mainly in the matrix. In [18] liver mitochondria contained ~8% of the total cellular calmodulin; mitochondrial calmodulin is mostly surface bound [11,13]. The role of mitochondrial calmodulin animals and plants is not yet known.

A large part of cellular calmodulin was localized outside organelles. The role of these calmodulin in plants is also not known, except that a part of plasma membrane-associated Ca^{2+}-ATPase is calmodulin-dependent and plays a role in the regulation of cytoplasmic $[Ca^{2+}]$ [4].

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