Subchloroplastic localization of NAD kinase activity: evidence for a Ca^{2+}, calmodulin-dependent activity at the envelope and for a Ca^{2+}, calmodulin-independent activity in the stroma of pea chloroplasts

Patrice Simon, Marc Bonzon, Hubert Greppin and Dieter Marmé*

Laboratoire de Physiologie Végétale, Université de Genève, 3 place de l'Université, CH-1211 Genève 4, Switzerland and **Institut für Biologie III, Universität Freiburg, Schänzlestr. 1, D-7800 Freiburg, FRG*

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Chloroplasts were prepared from pea seedlings and tested for NAD kinase activity. More than half of a Ca^{2+}, calmodulin-dependent activity and most of a Ca^{2+}, calmodulin-independent activity of the homogenate were associated with chloroplasts. The Ca^{2+}, calmodulin-dependent activity could be detected by adding Ca^{2+} and calmodulin to the incubation medium containing intact chloroplasts. This activity could not be separated from the chloroplasts by successive washes or by phase partition in aqueous two-polymer phase systems. After chloroplast fractionation, the Ca^{2+}, calmodulin-dependent NAD kinase activity was localized at the envelope, and the Ca^{2+}, calmodulin-independent activity was recovered from the stroma. In view of these results and of a previous report [Simon et al. (1982) Plant Cell Rep. 1, 119–122] the occurrence and presumed role of calmodulin in the chloroplast are discussed.

NAD kinase *Calmodulin* *Chloroplast envelope* *Pea chloroplast*

1. INTRODUCTION

The conversion of NAD to NADP is a light-dependent process observed in higher plant leaves [1–3] and chloroplasts [4]. NAD kinase (ATP:NAD 2′-phosphotransferase, EC 2.7.1.23) catalyzes the phosphorylation of NAD with ATP as phosphoryl donor [5]. With an enzyme preparation from pea seedlings authors in [6] observed that the enzyme required the presence of an activator, which was subsequently identified and characterized as the Ca^{2+}–calmodulin complex [7,8].

In several plants most of NAD kinase activity was shown to be located in the chloroplasts [4,9], but regulation by calmodulin was not investigated. In spinach, a chloroplastic and a cytoplasmic activity were demonstrated ([10], cf. also [5]); the chloroplastic activity represented only a small fraction of total activity and was independent of Ca^{2+} and calmodulin, whereas the cytoplasmic activity was very abundant and was dependent on Ca^{2+} and calmodulin. Furthermore, only a very low amount of calmodulin was detected in the chloroplasts [10]. This was confirmed by others [9,11]. Regulation of NAD kinase by Ca^{2+} and calmodulin was also demonstrated in dark-grown plants: in zucchini, NAD kinase activity was almost entirely located as a soluble enzyme in the cytoplasm [12] and in corn coleoptiles the activity was associated with the outer mitochondrial membrane and under the control of cytoplasmic Ca^{2+} and calmodulin [13]. Recently it was proposed that NAD kinase is a chloroplastic enzyme, located in the stroma and regulated by chloroplastic Ca^{2+}

Abbreviations: CaM, calmodulin; Chl, chlorophyll; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; flu, fluphenazine; GPD, glyceraldehyde-3-phosphate dehydrogenase

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and calmodulin [14]. Activation of chloroplastic NAD kinase by calmodulin was also mentioned [11].

The apparent discrepancy of NAD kinase compartmentation and regulation by Ca^{2+} and calmodulin could be due to interspecies variations [4]. We therefore reexamined the subcellular distribution and regulation of NAD kinase activity in pea seedlings, the same plant material used by the other research groups [4,11,14].

2. MATERIALS AND METHODS

2.1. *Chemicals*

Percoll™ and Dextran T500 were purchased from Pharmacia (Uppsala), PEG 4000 from Sigma (St. Louis, MO), glucose-6-phosphate dehydrogenase and phosphoglycerate kinase from Boehringer (Mannheim), fluphenazine from Von Heyden (Regensburg) and UDP-[^{14}C]galactose from the Radiochemical Centre (Amersham). Calmodulin was prepared from spinach according to [15]. All other reagents were of the highest grade.

2.2. *Plant material*

Pea seedlings (*Pisum sativum* L. cv. Alaska and cv. Petit Provençal) were grown in Vermiculite and nutrient solution in controlled environment chambers under an 8 h light : 16 h dark cycle. Light was provided at an illuminance of 6000 lx by fluorescent tubes. Temperature was maintained constant at 20°C. Humidity was 50% during the light and 70% during the dark period.

2.3. *Isolation and purification of intact chloroplasts*

Pea seedlings, 2–3 weeks old, were harvested during the first hour of the light period. Chloroplasts were extracted and purified on Percoll density gradients as in [10]. They were also purified by partition in two-polymer phase system using a batch procedure [16]. All manipulations were performed at 1–2°C.

2.4. *Preparation of a soluble fraction and a post-chloroplastic sediment*

The supernatant which was recovered after sedimentation of the chloroplasts in the pea homogenate was centrifuged at 50000 rpm for 30 min (Beckman Type 50). The resulting supernatant is referred to hereafter as the soluble fraction, and the pellet as the post-chloroplastic sediment.

2.5. *Preparation of chloroplast components*

All the solutions for preparing the chloroplast components were buffered with 10 mM Tricine–NaOH (pH 7.6) and contained 4 mM $MgCl_2$ [17,18]. After chloroplast lysis by a 3 min osmotic shock the suspension was adjusted to 0.3 M sucrose. Thylakoids were pelleted by centrifugation at 5000 rpm for 3 min (Beckman JA-21). The supernatant, which contained the stromal proteins, the envelopes and some thylakoids, was centrifuged at 50000 rpm for 20 min (Beckman Type 50). The supernatant was considered hereafter as the stroma fraction. The pellet was resuspended in 1 ml 0.3 M sucrose and layered on a 16-ml linear 0.5–1.5 M sucrose gradient. After 1 h centrifugation at 28000 rpm (Beckman SW 28.1), the gradient was eluted in 0.6 ml fractions.

2.6. *Assays*

NAD kinase activity was measured by a two-step procedure [4], slightly modified: incubation medium contained, in a total volume of 200 μl, 0.1 M Tris–HCl (pH 8), 2 mM NAD, 3 mM ATP and 6 mM $MgCl_2$, supplemented as indicated with 1 mM EGTA, 1 mM $CaCl_2$, 2 μg calmodulin/ml or 0.1 mM fluphenazine. Reaction was carried out at 37°C and stopped in a boiling water bath for 2 min. NADP was determined fluorimetrically after transformation to NADPH by glucose-6-phosphate dehydrogenase (6 nkat/ml) in 0.1 M Tris–HCl (pH 8) containing 0.2 mM glucose 6-phosphate. NADP-GPD was determined spectrophotometrically by measuring oxidation of NADPH in a test medium containing 50 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 4 mM EDTA, 3 mM ATP, 0.1 mM NADPH, 5 mM 3-phosphoglycerate, 10 μg phosphoglycerate kinase/ml (~7 μkat/mg). The ATPase activity was tested in 400 μl of incubation medium containing 50 mM Tricine-NaOH (pH 7.6), 5 mM $MnCl_2$ and 2.5 mM ATP; the reaction was stopped with 10% trichloroacetic acid after 30 min incubation at 37°C, and phosphate was estimated as in [19]. Galactosyltransferase activity was assayed as in [20], at a higher pH (7.6) and $MgCl_2$ concentration (4 mM)

[18], and at a lower specific activity of UDP-[^{14}C]galactose (22 kBq/ml). Activity was determined after 10 min incubation at 20°C. Proteins were estimated by a dye-binding assay [21] using reagent and standard from Bio-Rad (Munich). Chlorophyll was measured as in [22].

3. RESULTS

3.1. *Subcellular distribution of NAD kinase activity*

NAD kinase activity was determined in the various fractions obtained during chloroplast isolation and was found to depend on Ca^{2+} and calmodulin to a variable extent (table 1). In all fractions enzyme activity could be detected after chelating endogenous Ca^{2+}, but was maximal when incubation medium contained $CaCl_2$ and calmodulin. Addition of $CaCl_2$ alone caused a significant increase in enzyme activity, except in the purified chloroplast fraction. Addition of calmodulin further increased NAD kinase activity, and this stimulation was particularly evident in the purified chloroplast fraction. Activation by $CaCl_2$ was counteracted by the antipsychotic drug fluphenazine, which is known as a calmodulin antagonist [23]. Complete inhibition was already reached with 0.05 mM fluphenazine in the soluble fraction, whereas concentrations higher than 0.1 mM were necessary in the fractions which contained membranous material (not shown). These observations suggest that the effect of Ca^{2+} is probably linked to endogenous calmodulin. The NAD kinase activity measured in the absence of Ca^{2+} is referred to as the Ca^{2+}, calmodulin-independent activity, and the additional activity measured in the presence of $CaCl_2$ and an excess of calmodulin as the Ca^{2+}, calmodulin-dependent activity.

The results presented in table 1 were taken from one representative experiment, which was repeated more than 10 times, with qualitatively the same results. There was no difference between two varieties of peas tested. In the particular experiment shown in table 1 recovery of chloroplasts, as estimated by the chlorophyll content, was 10.5%, while recovery of Ca^{2+}, calmodulin-dependent and independent NAD kinase activities were 4.9 and 8.0%, respectively. These data indicate that about half of Ca^{2+}, calmodulin-dependent activity and most of Ca^{2+}, calmodulin-independent activity were associated to chloroplasts. The rest of NAD kinase activity was distributed in the soluble fraction and in the post-chloroplastic sediment.

3.2. *Characterization of chloroplastic NAD kinase activity*

Localization in the chloroplast of NAD kinase activity was investigated by measuring the enzyme activity before and after osmotic shock (latency test), assuming that the chloroplast envelope is impermeable to the substrates [24,25]. NADP-GPD was also determined as a marker of the stroma. A high Ca^{2+}, calmodulin-dependent NAD kinase activity was already detected with intact chloroplasts; after lysis, an additional activity independent of Ca^{2+} and calmodulin could be measured (table 2).

Table 1

NAD kinase activity of various subcellular fractions

Fraction	Protein (mg)	Chl (mg)	NAD kinase activity (nkat)				
			(1) EGTA	(2) $CaCl_2$	(3) $CaCl_2$ flu	(4) $CaCl_2$ CaM	(4) − (1)
Homogenate	1356	32.77(100)	3.87(100)	12.71	5.01	14.51	10.64(100)
Soluble	651	n.s.	0.34(8.8)	1.93	0.36	2.02	1.68(15.8)
Purified chloroplasts	101	3.43(10.5)	0.31(8.0)	0.41	0.36	0.83	0.52(4.9)
Post-chloroplastic sediment	408	5.62(17.1)	0.67(17.3)	3.47	1.22	3.91	3.24(30.5)

n.s., not significant. Test medium was supplemented as indicated with 1 mM EGTA, 1 mM $CaCl_2$, 0.1 mM fluphenazine and 2 μg calmodulin/ml. Percentage is given in brackets. Experiment started with 100 g of pea seedlings

Table 2

NAD kinase and NADP-GPD activities of intact and of osmotically lysed chloroplasts

Chloroplast state	Enzyme activity			
	NAD kinase (pkat/mg Chl)			NADP-GPD (nkat/mg Chl)
	(1) EGTA	(2) $CaCl_2$ CaM	(2) − (1)	
Intact	10	145	134	3.5
Lysed	54	189	135	26.1

Test medium was supplemented as indicated (cf. table 1)

Thus the activatable form of NAD kinase activity seems to be located at the outside of the chloroplast, probably on the outer face of the envelope, as it was accessible to calmodulin (18 kDa), whereas the non-activatable form is located inside, probably in the stroma.

The possibility of unspecific binding of Ca^{2+}, calmodulin-dependent NAD kinase activity was tested by varying the composition of homogenization solution. The presence of 1 mM $MgCl_2$ and/or $CaCl_2$, of 1 mM EDTA and/or EGTA, and of 0.1 M KCl neither abolished nor lowered the enzyme activity, and variations of pH from 6 to 8 were also without effect (not shown). Furthermore, chloroplasts were submitted to successive washes. NAD kinase and NADP-GPD activities were measured after each wash and computed as enzyme activity ratios (fig.1). Washing did not lower the Ca^{2+}, calmodulin-independent activity, as expected, nor the Ca^{2+}, calmodulin-dependent activity, which appeared therefore closely bound to the chloroplast envelope. When chloroplasts were further purified by partition in aqueous two-polymer phase system, the Ca^{2+}, calmodulin-dependent NAD kinase activity followed strictly the distribution pattern of NADP-GPD (fig.2).

3.3. *Subchloroplastic localization of NAD kinase activity*

After sedimentation of a crude envelope preparation in a sucrose density linear gradient, 3 bands were visualized: a brownish-orange band at the upper zone (I), a yellowish one in the middle

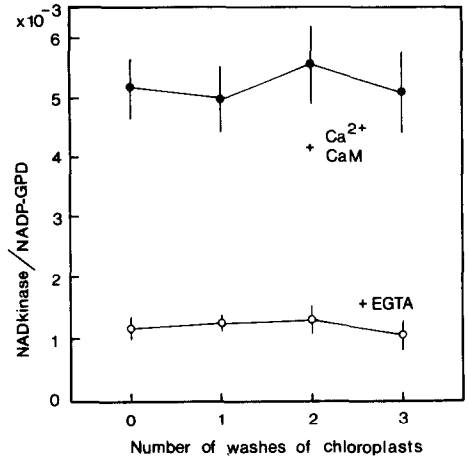

Fig.1. Effect of washing on NAD kinase activity of chloroplast preparations. After each wash, chloroplasts were tested for NAD kinase and NADP-GPD activities. Test medium for NAD kinase activity contained 1 mM EGTA (○) or 1 mM $CaCl_2$ and 2 μg CaM/ml (●). Bars represent means ± SD of 3 replicate experiments.

part (II) and a greenish one at the bottom (III), corresponding to thylakoids (fig.3). Envelopes were localized in a buoyant density range of 1.082–1.144 (maximum at 1.113) by measuring activities of the marker enzymes, galactosyltransferase and Mn^{2+}-ATPase [17,26,27]. Ca^{2+}, calmodulin-dependent NAD kinase activity showed the same density profile as the envelope markers activities. Some ATPase and Ca^{2+}, calmodulin-independent NAD kinase activities were recovered with band I. Sedimentation of this band was not isopycnic, and its exact nature has not been elucidated. It was composed of low-M_r polypeptides, and traces of envelope carotenoids were detected; it was not affected by inclusion in the homogenization solution of protease inhibitors, e.g., 0.4 mM phenylmethylsulfonyl fluoride and 10 mM benzamidine (not shown).

As shown in table 3 most of the NAD kinase activity is associated with the envelope. Addition of Ca^{2+} caused only a slight increase of the activity whereas addition of Ca^{2+} and calmodulin resulted in a 45-fold stimulation. In contrast, the stroma associated NAD kinase activity was only slightly increased by Ca^{2+} and calmodulin. In both cases the calmodulin antagonist fluphenazine inhibited the Ca^{2+}, calmodulin-dependent activity. Thylakoids contained only traces of activity after several washes (not shown).

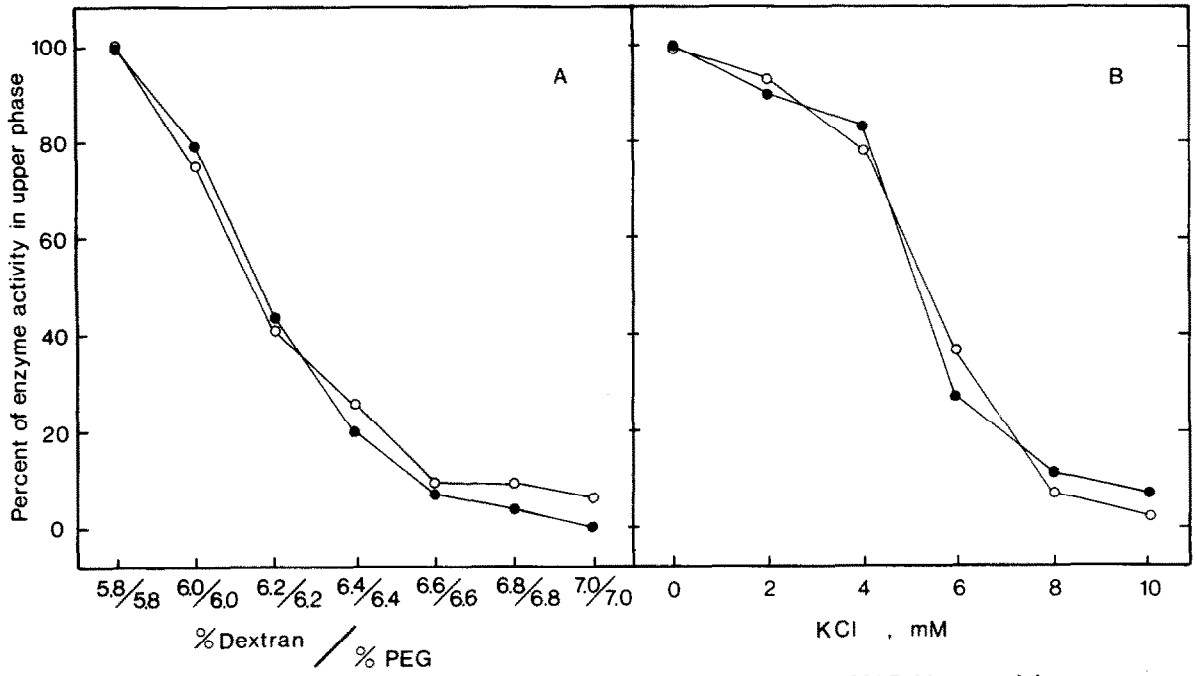

Fig.2. Phase partition of intact chloroplasts in aqueous two-polymer phase system. NAD kinase activity was measured in the presence of 1 mM $CaCl_2$ and 2 μg CaM/ml (●). NADP-GPD (○) was used as a marker of intact chloroplasts (stroma). (A) Effect of increasing polymer concentration. Each system contained chloroplasts equivalent to 0.48 mg Chl and 12.9 mg protein, with 86.4 pkat of NAD kinase activity and 17.4 nkat of NADP-GPD activity. (B) Effect of increasing salt concentration. Each system contained 5.9% (w/w) polymer and chloroplasts equivalent to 0.27 mg Chl and 4.48 mg protein, with 37.2 pkat of NAD kinase activity and 7.29 nkat of NADP-GPD activity.

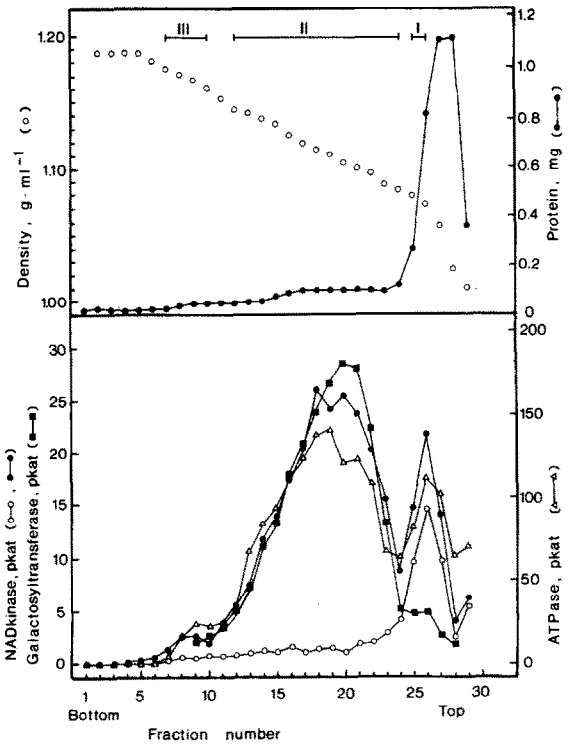

Table 3

NAD kinase activity of the chloroplast components

Chloroplast component	NAD kinase activity (pkat/mg protein)			
	EGTA	$CaCl_2$	$CaCl_2$ CaM	$CaCl_2$ CaM flu
Stroma	4.9	5.4	5.5	5.1
Envelope	11.5	27.2	250.1	16.1

Test medium was supplemented as indicated (cf. table 1). Fractionation of chloroplasts equivalent to 3.8 mg Chl and 118 mg protein yielded 77.6 mg of stromal protein and 0.89 mg of envelope protein

Fig.3. Fractionation of chloroplast envelopes on a continuous sucrose density gradient. A crude envelope preparation (1 ml, 5.3 mg protein), which was obtained from 4.3 mg Chl of intact chloroplasts, was sedimented on a 16-ml linear 0.5–1.5 M sucrose gradient. Incubation medium for NAD kinase activity contained 1 mM EGTA (○) or 1 mM $CaCl_2$ and 2 μg CaM/ml (●).

4. DISCUSSION

The results presented here confirm that NAD kinase activity in pea is essentially associated with chloroplasts [4] partially located in the stroma and partially at the envelope. However, as in spinach [10], the stromal NAD kinase activity is not Ca^{2+}, calmodulin-dependent, whereas the envelope-bound activity is Ca^{2+}, calmodulin-dependent. The activity detected in the post-chloroplastic sediment is probably associated with released chloroplast envelopes, or with mitochondria [13].

As pointed out in [10], the independence of the stromal NAD kinase activity of Ca^{2+} and calmodulin is not in agreement with the hypothesis in [14] that all NAD kinase in plants is chloroplastic and that the light-induced conversion of NAD to NADP [4] is mediated by chloroplastic Ca^{2+} and calmodulin. Cellular localization of Ca^{2+}, calmodulin-dependent NAD kinase activity varies among different plant species. Most of the enzyme activity is pelletable and essentially associated with the chloroplast envelope in pea (here) and with the outer mitochondrial membrane in dark-grown corn coleoptiles [13]. In zucchini [12] and in spinach [10] most of the NAD kinase activity is soluble and located in the cytoplasm. In all cases NAD kinase is freely accessible to cytoplasmic free Ca^{2+} and calmodulin.

If we consider our findings that stromal NAD kinase activity is not regulated by Ca^{2+} and calmodulin and that only low amounts of calmodulin have been found in chloroplasts [9–11], the regulatory role of Ca^{2+} and calmodulin in the chloroplast compartment and on photosynthesis [11,14] has to be questioned seriously. Recently it was reported that calmodulin-binding proteins are found in the envelope but not in the thylakoids and in the stroma of pea and spinach chloroplasts [29]. All these observations do not favor the hypothesis that calmodulin is directly in chloroplastic activities [14]. However, they are in agreement with the assumption that soluble and particulate NAD kinase activity is regulated by the free cytoplasmic Ca^{2+} concentration which has been found to be under the control of light [28]. The mechanism by which chloroplastic NAD kinase seems to be photoactivated [4] remains to be determined.

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REFERENCES

[1] Ogren, W.L. and Krogmann, D.W. (1965) J. Biol. Chem. 240, 4603–4608.
[2] Graham, D. and Cooper, J.E. (1967) Aust. J. Biol. Sci. 20, 319–327.
[3] Bonzon, M., Simon, P., Greppin, H. and Wagner, E. (1983) Planta 159, 254–260.
[4] Muto, S., Miyachi, S., Usada, H., Edwards, G.E. and Bassham, J.A. (1981) Plant Physiol. 68, 324–328.
[5] Yamamoto, Y. (1966) Plant Physiol. 41, 523–528.
[6] Muto, S. and Miyachi, S. (1977) Plant Physiol. 59, 55–60.
[7] Anderson, J.M. and Cormier, M.J. (1978) Biochem. Biophys. Res. Commun. 84, 595–602.
[8] Anderson, J.M., Charbonneau, H., Jones, H.P., McCann, R.O. and Cormier, M.J. (1980) Biochemistry 19, 3113–3120.
[9] Muto, S. (1982) FEBS Lett. 147, 161–164.
[10] Simon, P., Dieter, P., Bonzon, M., Greppin, H. and Marmé, D. (1982) Plant Cell Rep. 1, 119–122.
[11] Jarrett, H.W., Brown, C.J., Black, C.C. and Cormier, M.J. (1982) J. Biol. Chem. 257, 13795–13804.
[12] Dieter, P. and Marmé, D. (1980) Cell Calcium 1, 279–286.
[13] Dieter, P. and Marmé, D. (1983) J. Biol. Chem., in press.
[14] Cormier, M.J., Jarrett, H.W. and Charbonneau, H. (1982) in: Calmodulin and Intracellular Ca^{2+} Receptors (Kakiuchi, S. et al. eds) pp.125–139, Plenum, New York.
[15] Watterson, D.M., Iverson, D.B. and Van Eldik, L.J. (1980) Biochemistry 19, 5762–5768.
[16] Larsson, C. and Anderson, B. (1979) in: Plant Organelles (Reid, E. ed) Methodological Surveys: Biochemistry, vol.9, pp.35–46, Ellis Horwood, Chichester.
[17] Douce, R. and Joyard, J. (1979) Adv. Bot. Res. 7, 1–116.
[18] Cline, K., Andrews, J., Mersey, B., Newcomb, E.H. and Keegstra, K. (1981) Proc. Natl. Acad. Sci. USA 78, 3595–3599.
[19] Chen, P.S., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756–1758.

[20] Douce, R. and Joyard, J. (1980) Methods Enzymol. 69C, 290–301.
[21] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[22] Bruinsma, J. (1961) Biochim. Biophys. Acta 52, 576–578.
[23] Levin, R.M. and Weiss, B. (1979) J. Pharmacol. Exp. Ther. 208, 454–459.
[24] Heber, U. and Santarius, K.A. (1965) Biochim. Biophys. Acta 109, 390–408.
[25] Heber, U. and Santarius, K.A. (1970) Z. Naturforsch. 25b, 718–728.
[26] Douce, R. (1974) Science 183, 852–853.
[27] Joyard, J. and Douce, R. (1975) FEBS Lett. 51, 335–340.
[28] Marmé, D. and Dieter, P. (1983) in: Calcium and Cell Function (Cheung, W.Y. ed) vol.IV, pp.263–311, Academic Press, New York.
[29] Roberts, D.M., Zielinski, R.E., Schleicher, M. and Watterson, D.M. (1983) J. Cell Biol. 97, 1644–1647.