Light-Induced Conversion of Nicotinamide Adenine Dinucleotide to Nicotinamide Adenine Dinucleotide Phosphate in Higher Plant Leaves[1]

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ABSTRACT

Light-induced conversion of NAD to NADP was investigated in higher plants. Upon illumination, conversion of NAD to NADP was observed in intact leaves of wheat and pea following incubation in the dark. This conversion was also observed in mesophyll protoplasts of wheat leaves when they were isolated in the dark or isolated in light and then preincubated in the dark. Chloroplasts isolated from wheat protoplasts prepared in the dark carried out the conversion. The conversion in the mechanically isolated spinach chloroplasts was observed only when they were isolated in the dark from leaves preincubated in darkness.

Sucrose density gradient centrifugation of wheat protoplast extracts and differential centrifugation of protoplast extracts from various plants showed that most of the NAD kinase was localized in the chloroplasts. Therefore, the conversion of NAD to NADP is considered to occur in the chloroplasts. However, with extracts of maize mesophyll protoplasts, the enzyme was localized in the extrachloroplast fraction. The NAD kinase was activated some 30% by illumination of leaves or protoplasts of pea and wheat after preincubation in the dark.

These results suggest that, in general, the light-induced conversion of NAD to NADP occurs in the chloroplast and is catalyzed by photoactivated NAD kinase using photochemically produced ATP.

The light-induced conversion of NAD to NADP was first discovered in *Chlorella* cells by Oh-hama and Miyachi (20), and a similar conversion was reported later with higher plant leaves (6, 19). Recently, the conversion reaction in *Chlorella* cells was extensively studied in relation to photosynthesis (Matsumura-Kadota, Muto, and Miyachi, in preparation). The conversion of NAD to NADP was saturated with relatively low light intensity.

Either red or blue light, but not green or far-red light, was effective. CMU, disalicylidene propanediamine, and carbonyl-cyanide 3-chlorophenylhydrazone were effective inhibitors for the conversion reaction. These results indicate the association of photosynthetic electron transport and photophosphorylation in this conversion reaction. Since NAD kinase is the only enzyme which is known to catalyze the formation of NADP from NAD, it was proposed that this enzyme phosphorylated NAD using photochemically produced ATP. Oh-hama *et al.* (21) also suggested the photoactivation of NAD kinase.

Determination of the intracellular location of the conversion of NAD to NADP is very important for the understanding of the physiological role of the conversion. Ogren and Krogman (19) fractionated lyophilized leaves by a nonaqueous method and found that the NADP formed by illumination was localized in the chloroplast. However, most of the NAD kinase activity was recovered outside the chloroplast.

The present study was conducted to learn (a), whether the light-induced conversion of NAD to NADP occurs inside or outside the chloroplast in leaf cells; (b), whether NAD kinase actually located outside the chloroplast; and (c), whether NAD kinase is photoactivated or not.

MATERIALS AND METHODS

Plant Material and Protoplast Preparation. Pea (*Pisum sativum* L.) was grown in a greenhouse (18); wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) were grown in growth chambers (25), and spinach (*Spinacia oleracea* L.) was grown in a growth chamber (10), as previously described. Crabgrass (*Digitaria adscendens* Henr.), foxtail (*Setaria viridis* Beauv.), and sunflower (*Helianthus annuus* L.) were harvested from gardens at the University of Wisconsin, Madison, or the University of Tokyo, Tokyo.

The preparation of protoplasts was carried out under illumination with fluorescent lamps or in the dark using a dim green light when necessary. Mesophyll cell protoplasts of C_4 and C_3 plants, except pea, were enzymically isolated and purified as described (4, 5, 11) using a combination of Cellulase R-10 and Macerozyme R-10 (Kinki Yakult Mfg., Nishinomiya, Japan). The preparations were suspended in a medium consisting of 0.4 M sorbitol, 50 mM Hepes-KOH (pH 7.6), 10 mM NaHCO₃, and 1 mM EDTA.

Pea mesophyll cell protoplasts also were prepared enzymically. The digestion medium contained 0.6 M sorbitol, 2 mM MgCl₂, 1 mM KH₂PO₄, 2%(w/v) Cellulase R-10, 0.2%(w/v) Macerozyme R-

10, and 10 mM Mes-KOH (pH 5.5). Isolated protoplasts were purified by an aqueous liquid-liquid two-phase method (12) and suspended in 0.4 M sorbitol containing 50 mM Hepes-KOH (pH 7.6), 1 mM MgCl₂, and 2 mM KH₂PO₄.

Preparation of Chloroplasts. Wheat chloroplasts were isolated from ruptured protoplasts as described (4) and suspended in a medium consisting of 50 mM Hepes-KOH (pH 7.6), 0.4 M sorbitol, 10 mM NaHCO₃, 1 mM EDTA, and 0.3 mM KH₂PO₄. Spinach chloroplasts were prepared and suspended according to the method of Jensen and Bassham (9).

Intactness of chloroplasts was tested by ferricyanide-dependent O₂ evolution before and after osmotic shock, as described (4, 14). According to this criterion of intactness, chloroplasts isolated from protoplasts and spinach leaves were greater than 90 and 80% intact, respectively.

Sucrose Density Gradient Centrifugation. Protoplasts were ruptured by passing through 20-μm nylon net, layered on a sucrose gradient, and centrifuged (25) using a gradient similar to that of Miflin and Beevers (17). After centrifugation, the gradient was fractionated into 1.2-ml fractions. Sucrose concentration was determined by refractometry.

Enzyme Assay and Chl Determination. NADP triose-P dehydrogenase, alcohol dehydrogenase, hydroxypyruvate reductase, and fumarase were assayed as previously described (25). The reaction mixture of NAD kinase (18) contained 0.1 mM CaCl₂. The reaction product NADP was assayed as described below.

Chl was determined by the method of Wintermans and De Mots (26). Protein was measured by a dye-binding method (2).

Extraction and Assay of Nicotinamide Coenzymes. Lyophilized plants were ground in a mortar with either 0.1 N NaOH (for reduced forms) or 0.1 N HCl (for oxidized forms) and heated for 2 min in a boiling waterbath. After immediate cooling, homogenates were centrifuged at 15,000g for 10 min. The resulting supernatant solutions were used for assay of nicotinamide coenzymes. Either 1 N HCl or 1 N NaOH was added to suspensions of protoplasts or chloroplasts to make a final concentration of 0.1 N. The resulting mixture was heated for 2 min and treated as above. An enzymic cycling method described (15) was used to measure nicotinamide coenzymes. Recoveries of NAD, NADH, NADP, and NADPH added externally to lyophilized plants were 113.6 ± 4.1, 94.5 ± 5.9, 117.5 ± 5.4, and 74.1 ± 2.1%, respectively.

RESULTS

Light-Induced Conversion of NAD to NADP. When wheat leaves were illuminated after the dark overnight incubation at 25 C, NAD decreased and NADP increased for the first 5 min. Reduced coenzymes increased slightly. The level of NAD-plus-NADH decreased from 73.5 to 56.0%, and that of NADP-plus-NADPH increased from 26.5 to 44.0% after 15 min (Fig. 1). Light-induced decrease in NAD and increase in NADP were also observed with pea seedlings (Table I). Levels of the reduced coenzymes were much lower than those of the oxidized forms. The decrease in the amount of NAD-plus-NADH was approximately the same as the increase in the amount of NADP-plus-NADPH, indicating the light-induced conversion of NAD to NADP in pea seedlings.

Wheat protoplasts were prepared under illumination. The protoplasts were incubated in the dark for 60 min and then illuminated for 15 min, and the levels of nicotinamide coenzymes were measured (Fig. 2). During dark incubation prior to illumination, the total nicotinamide coenzyme level rapidly decreased in the first 15 min and, thereafter, remained constant. During this period, NADP-plus-NADPH decreased rapidly, while NAD-plus-NADH increased slowly. We, therefore, infer that there occurred a rapid breakdown of NADP and NADPH to some unknown compound(s) other than NAD during the initial dark period. In light, NAD and NADH decreased while NADP and NADPH increased.
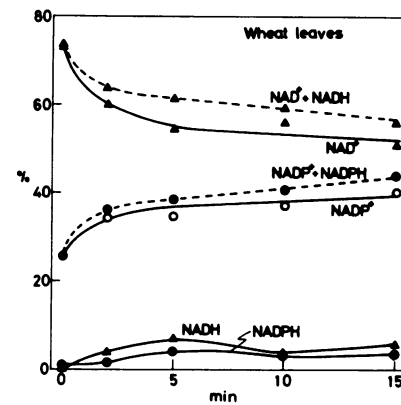

FIG. 1. Changes of nicotinamide coenzyme levels in intact wheat leaves by illumination. Twelve-day-old wheat plants were kept in the dark overnight at 25 C. Leaves were excised, floated on distilled H₂O, and illuminated at 200,000 erg/cm²·s by incandescent lamps. The leaves were plunged into liquid N₂ and lyophilized. Lyophilized leaves were extracted, and nicotinamide coenzymes were assayed, as described in "Materials and Methods." (△——△), NAD; (▲——▲), NADH; (○——○), NADP; (●——●), NADPH; (▲- - -▲), NAD-plus-NADH; (●- - -●), NADP-plus-NADPH.

The time course of decrease of NAD-plus-NADPH was a mirror image of that of the increase of NADP-plus-NADPH. The total nicotinamide coenzyme level remained almost constant during illumination; therefore, NAD was considered to be converted to NADP. The amount of NADP formed from NAD (differences of steady state levels between dark and light) varied from 3 to 9 nmol/mg Chl. When wheat protoplasts were isolated in the dark, similar light-induced changes of nicotinamide coenzymes were observed without dark preincubation (data not shown). These changes in nicotinamide coenzymes are similar to those observed with *Chlorella* cells (20) (Matsumura-Kadota, Muto, Miyachi, in preparation).

To investigate the intracellular location of the light-induced conversion of NAD to NADP, the effect of light on the levels of nicotinamide coenzymes was studied with intact chloroplasts isolated in the dark from wheat protoplasts which had been prepared in the dark. Illumination caused an increase of NADP-plus-NADPH and a decrease of NAD-plus-NADH (Fig. 3). Time courses of the decrease and the increase were mirror images of each other while the total level was kept relatively constant. This indicates that a conversion of NAD to NADP occurred in chloroplasts. However, there was a lag phase which has not been observed with protoplasts or intact leaves.

The conversion of NAD to NADP also occurred in spinach chloroplasts isolated from leaves which had been preincubated in the dark (data not shown). No such conversion was observed without dark preincubation. Time courses of increase in NADP-plus-NADPH and decrease in NAD-plus-NADH were similar to those observed in wheat chloroplasts. However, NADP rapidly decreased upon illumination and then increased gradually while NADPH rapidly increased at first and then remained at a constant level. As a result, the ratio of NADPH to NADP rose quickly from 0.3 to 4 and then remained higher than 2 during illumination. The amount of NADP formed from NAD varied from 2 to 5 nmol/mg Chl. No such pronounced light-induced shift in the ratio of NADPH to NADP was observed in wheat chloroplast or other plant leaves.

Subcellular Localization of NAD Kinase. Figure 4 shows the localization of several enzymes on a sucrose density gradient following centrifugation and fractionation of a wheat protoplast extract. Most of the NAD kinase was contained in the intact chloroplast fraction, but some of the activity was in the cytoplas-

Table I. *Light-Induced Changes of Nicotinamide Coenzyme Levels in Pea Seedlings*

Pea seedlings (11-day-old) were illuminated for 15 min at 12,000 erg/cm$^2\cdot$s and 23 C with day-light fluorescent lamps after dark preincubation overnight at 23 C.

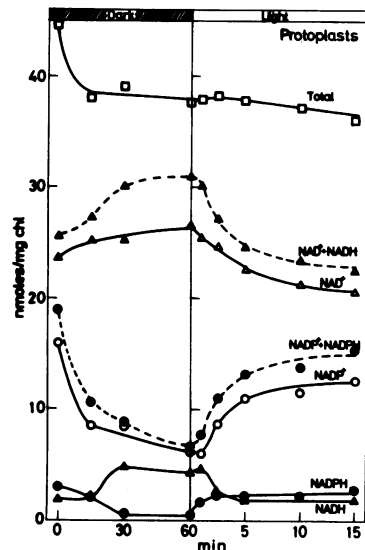|	NAD	NADH	NAD + NADH	NADP	NADPH	NADP + NADPH	Total
			nmol/100 mg dry wt				
Dark	39.96	3.84	43.80	13.08	2.04	15.12	58.92
Light	29.52	3.36	32.88	22.88	3.50	26.38	59.26
Light—Dark	−10.44	−0.48	−10.92	9.80	1.46	11.26	0.34

FIG. 2. Changes of nicotinamide coenzyme levels in wheat protoplasts by illumination. Wheat leaves were digested under illumination of fluorescent lamps at 10,000 erg/cm$^2\cdot$s, as described in "Materials and Methods." Suspensions of purified protoplasts (0.9 ml) were incubated in test tubes (1.2 × 10 cm) for 30 min in the dark and then illuminated from the bottom by incandescent lamps at 200,000 erg/cm$^2\cdot$s. During the experiment, tubes were occasionally shaken gently to prevent precipitation of protoplasts. The temperature was 25 C. Symbols are as in Figure 1; (□——□), total nicotinamide coenzymes.
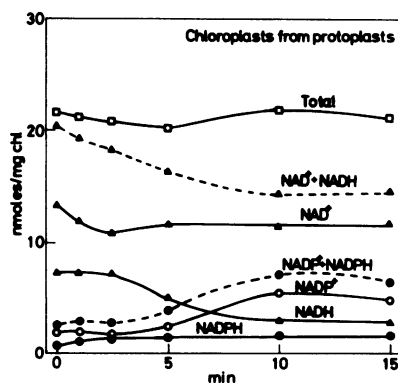

FIG. 3. Changes of nicotinamide coenzyme levels of wheat chloroplasts. Chloroplasts were isolated in the dark from wheat protoplasts which were prepared in the dark. After dark preincubation for 5 min, chloroplasts were illuminated as described in Figure 2. Symbols are as in Figure 2.

mic fraction (top of the gradient). The distribution profile of NAD kinase was similar to that of NADP triose-P dehydrogenase, a marker enzyme for the chloroplast stroma. The intact chloroplast fraction had some contamination by peroxisomes but none by mitochondria or cytoplasm, as judged by distribution of marker enzymes in the respective fractions. These results indicate that
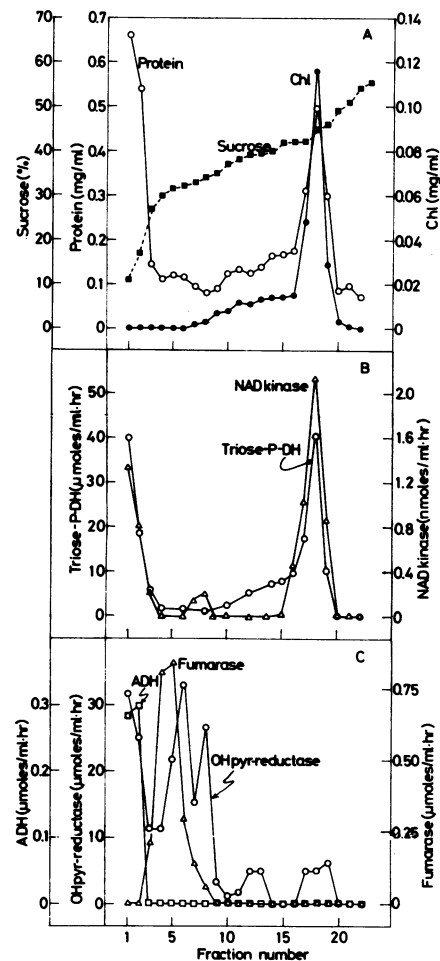

FIG. 4. Enzyme distribution after separation of (a) wheat protoplast extract on a sucrose density gradient. The ruptured protoplast preparation (2 ml) was layered on top of the gradient, which was prepared as previously described (25), and centrifuged at 4,000 rpm for 5 min, then at 10,000 rpm for an additional 10 min in a Beckman JS 13 rotor. ADH, alcohol dehydrogenase (cytoplasmic marker); OH pyr-reductase, hydroxypyruvate reductase (peroxisomal marker); triose-P-DH, NADP triose-P dehydrogenase (intact chloroplast marker).

most of the NAD kinase is localized in the chloroplasts with little, if any, in the cytoplasm and none in the mitochondria or peroxisomes.

The subcellular location of NAD kinase was tested in several other plants by differential centrifugation of mesophyll protoplast extracts (Table II). The distribution of the enzyme *versus* NADP triose-P dehydrogenase indicated that most of the NAD kinase was localized in the chloroplasts of all plants tested except maize. In maize mesophyll protoplasts, the NAD kinase was found mostly outside the chloroplasts. Although the results shown were obtained with maize grown in a growth chamber, similar results were

Table II. *Intracellular Distribution of NAD Kinase and NADP Triose-P Dehydrogenase in Mesophyll Protoplasts of Various Plants*

Protoplasts, ruptured by passing through a 20-μm nylon cloth, were centrifuged at 650g for 1 min to precipitate chloroplasts. Pellets were resuspended in an equivalent volume of the same medium as used for protoplast suspension (see "Materials and Methods").

Enzyme	Plant	Chloroplast	Supernatant
		nmol/h·ml enzyme	
NAD kinase	Wheat	11.2 (94.7)[a]	0.63 (5.3)
	Pea	26.4 (71.9)	10.3 (28.1)
	Sunflower	8.55 (94.7)	0.48 (5.3)
	Crabgrass	10.6 (86.3)	1.68 (13.7)
	Foxtail	2.79 (87.5)	0.40 (12.5)
	Maize	0.10 (5.3)	1.79 (94.7)
		μmol/min·ml enzyme	
NADP-triose-P	Wheat	2.16 (97.8)	0.048 (2.2)
dehydrogenase	Pea	2.86 (74.7)	0.970 (25.3)
	Sunflower	1.98 (98.0)	0.040 (2.0)
	Crabgrass	3.27 (88.1)	0.442 (11.9)
	Foxtail	4.14 (82.4)	0.884 (17.6)
	Maize	3.34 (85.8)	0.555 (14.2)

[a] Numbers in parentheses, percentage of total (chloroplast plus supernatant).

Table III. *Photoactivation of NAD Kinase in Pea Seedlings*

Pea seedlings (11-day-old) were homogenized with 50 mM Hepes-KOH buffer (pH 7.6) and 1% polyamide 11 in a mortar on ice. The homogenate was filtered through nylon cloth and centrifuged at 15,000g for 20 min at 4 C. The supernatant was used as the enzyme preparation.

	NAD Kinase Activity		
	Dark	Light	Activation
	nmol NADP/mg protein·h		*%*
Experiment 1[a]	21.2	27.0	27.4
Experiment 2[b]	19.8	27.6	39.4

[a] Pea seedlings, which had been preincubated in the dark overnight, were detached and floated on distilled H_2O at 25 C and illuminated at 200,000 erg/cm^2·s by incandescent lamps for 30 min.

[b] Pea seedlings planted in a tray were preincubated in the dark overnight, then illuminated at 120,000 erg/cm^2·s with day-light fluorescent lamps for 1 h at 23 C.

obtained with maize plants grown in a field.

Photoactivation of NAD Kinase. The effect of light on NAD kinase activity was examined with pea seedlings after dark preincubation overnight (Table III). When detached pea seedlings were floated on distilled H_2O and illuminated for 30 min at 200,000 erg/cm^2·s with incandescent lamps, there was a 27% increase in activity of the enzyme. Greater activation was obtained with attached seedlings by illumination at 120,000 erg/cm^2·s with fluorescent lamps for 1 h. Since the extraction procedure employed in these experiments was time consuming, it is possible that the light-activated NAD kinase was deactivated during extraction. To avoid this and study the time course of activation, leaf mesophyll protoplasts of pea were employed. Protoplasts were illuminated after dark preincubation. At various times after illumination, a sample of protoplast was added to the NAD kinase assay mixture. This allowed immediate assay of the enzyme following osmotic shock of the protoplasts. Maximum activity was reached within 1 min of illumination (Table IV). The magnitude of activation was almost the same as with intact detached plants. This result indicates that the enzyme remains fully activated during extraction.

Similar photoactivation was observed with wheat protoplasts (Table IV).

DISCUSSION

The present study clearly shows that the light-induced conversion of NAD to NADP can occur in isolated chloroplasts. The conversion in chloroplasts has been suggested by Ogren and Krogman (19) in their nonaqueous fractionation study of bean leaves. However, no such conversion has been reported from studies with isolated chloroplasts, although illumination was found to cause a rapid conversion of nicotinamide coenzymes to the reduced form (7, 8, 13). Lack of the conversion of NAD to NADP may be attributed to the fact that chloroplasts used in these previous studies were isolated without dark preincubation. The rapid light-induced reduction of NADP was observed only in the chloroplasts isolated from spinach. No such significant change was observed in wheat or *Chlorella*. The light-induced conversion of NAD to NADP was observed in wheat, pea, spinach (the present study), kidney bean, *Chlamydomonas* (19), mung bean (6), and *Chlorella* (20) (Matsumura-Kadota, Muto, Miyachi, in preparation). Therefore, it may be a common phenomenon in higher plants and green algae.

NAD kinase is assumed to catalyze the light-induced convesion of NAD to NADP (21). In the present study, the enzyme was shown to be localized mostly in chloroplasts and rapidly activated by illumination. The activity of activated enzyme (~30 nmol NADP/mg Chl·h) is sufficient to account for the amount of NADP converted from NAD during illumination for 15 min (3 to 9 nmol/mg Chl). Therefore, we propose that the light-induced conversion of NAD to NADP is the result of phosphorylation of NAD by photochemically produced ATP and that it is catalyzed by photoactivated NAD kinase in chloroplasts. The conversion started immediately after illumination in intact leaves or protoplasts, while a lag time was observed in isolated chloroplasts. The reason for this discrepancy is not clear at this moment.

During dark-light transition, starch metabolism in chloroplasts changes from degradation to biosynthesis. In light, chloroplasts assimilate CO_2 to form starch *via* the reductive pentose phosphate pathway. In the dark, only degradation of starch *via* the glycolytic and the oxidative pentose phosphate pathways occurs (10, 22, 24). The metabolic steps requiring nicotinamide coenzymes in the reductive pentose phosphate and the glycolytic pathways are catalyzed by triose-P dehydrogenases. The enzyme localized in chloroplasts is able to use both NAD and NADP as coenzymes (16). In the presence of NADP, the enzyme exists mainly as a protomer which has a high affinity for NADP and high NADP-dependent activity (NADP-dependent form). The enzyme aggre-

Table IV. *Photoactivation of NAD Kinase in Mesophyll Protoplasts of Wheat and Pea Leaves*

Protoplast suspension (100 μl) was incubated for 1.5 h in the dark at 25 C and illuminated at 20,000 erg/cm^2·s with incandescent lamps for the indicated time. The enzyme reaction was started by adding 400 μl of reaction mixture to the suspension. After 30 min at 25 C, the reaction was terminated by adding 100 μl 1 N HCl.

Time of Illumination	Enzyme Activity	
	Pea Protoplasts	Wheat Protoplasts
min	*nmol NADP/mg Chl*	
0	20.5 (100)[a]	19.2 (100)
0.5	24.4 (119)	18.3 (95)
1	26.7 (130)	23.6 (123)
2	26.0 (127)	23.5 (122)
5	26.5 (129)	22.7 (118)
10	26.7 (130)	24.4 (127)

[a] Numbers in parenthesis, percentage of activity at 0 min.

gates in the absence of NADP to form a tetramer which has a K_m for NADP considerably larger than that for NAD (NAD-dependent form) (23). These facts suggest that the enzyme exists as an equilibrium mixture of tetramer and protomer, depending on the levels of nicotinamide coenzymes. Therefore, the light-induced conversion of NAD to NADP in the chloroplasts may be responsible for the conversion of the triose-P dehydrogenase from NAD-dependent to NADP-dependent form. Consequently, the metabolic pathway in the chloroplasts may be switched from the glycolytic to the reductive pentose phosphate pathway in light. The NADP-dependent triose-P dehydrogenase, as well as fructosebisphosphatase, sedohepturosebisphosphatase, and phosphoribulokinase, is activated by ferredoxin/thioredoxin system in light (3). This accelerates the reductive pentose phosphate pathway. The oxidative pentose phosphate pathway is the other pathway participating in starch breakdown in chloroplasts, and it requires NADP for its operation. This would be the reason that the activity of NAD kinase is high even in the dark. In light, this pathway is suppressed because glucose 6-P dehydrogenase is photoinactivated (1) and inhibited by a high ratio of NADPH to NADP (13).

In most plants examined, the NAD kinase is localized in the chloroplasts. However, NAD kinase is localized in the cytoplasm of mesophyll cells of maize. Intact leaves of maize carried out the light-induced conversion of NAD to NADP (data not shown). Further studies are necessary to correlate the light-induced conversion of NAD to NADP with the metabolic requirement of NADP in both mesophyll and bundle sheath cells of various C_4 plants.

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