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Nicotinamide Adenine Dinucleotide Phosphate Photoreduction from Water by Agranal Chloroplasts Isolated from Bundle Sheath Cells of Maize

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ABSTRACT

Photoreduction of NADP from water in agranal chloroplasts isolated from the leaf bundle sheath cells of *Zea mays* (var. DS 606A) or *Sorghum bicolor* (var. Texas 610) was dependent upon addition of plastocyanin as well as ferredoxin. Activity was further increased by the addition of ferredoxin NADP-reductase. Saturation for plastocyanin was reached at about 6 micromolar. In contrast, grana-containing chloroplasts isolated from leaf mesophyll cells of these plants or from pea (*Pisum sativum* L.) leaves did not require either plastocyanin or ferredoxin NADP-reductase for NADP photoreduction from water, although with some preparations plastocyanin stimulated the activity.

Photosystem I activity, which was low in washed preparations of bundle sheath chloroplasts, was also stimulated by plastocyanin. The effect of plastocyanin on photosystem I activity in the grana-containing chloroplasts was similar to that on NADP photoreduction from water.

In the presence of plastocyanin, the rates of NADP photoreduction from water were about the same in the agranal and granal chloroplasts, but photosystem I activity was considerably higher in bundle sheath chloroplasts. In these chloroplasts photosystem II appeared to limit the rate of NADP photoreduction.

The results indicated that the agranal bundle sheath chloroplasts reduced plastocyanin via photosystem II and oxidized it via photosystem I. Both types of maize chloroplast photoreduced oxidized plastocyanin, but in the presence of methyl viologen, reduced plastocyanin was photo-oxidized only by the bundle sheath chloroplasts.

The agranal chloroplasts found in the bundle sheath cells of certain plants, including maize and *Sorghum*, when isolated from the cells do not have the capacity to photoreduce NADP (1, 4, 5, 25). In contrast the isolated grana-containing chloroplasts of the mesophyll cells of the same plants carry out a normal photoreduction of NADP. This difference between the two chloroplast types has been attributed to a deficiency of photosystem II (25) or to a deficiency of a component between photosystem II and photosystem I (5) in the isolated bundle sheath chloroplasts.

In an accompanying paper (6), evidence is presented for electron flow between photosystem II and cytochrome *f*, which is localized in photosystem I (8), in chloroplasts of the intact bundle sheath cell of maize. It has been reported that in another C4 plant, *Digitaria sanguinalis* (L) Scop., the isolated bundle sheath cells will photosynthetically fix CO_2 in the presence of ribulose-1,5-diP, or ribulose 5-P (9). These observations have led us to suggest (5) that isolated bundle sheath chloroplasts may lack one or more soluble components which are necessary to complete the electron transfer chain between photosystem II and the reduction of NADP via photosystem I.

In this paper we show that maize bundle sheath chloroplasts will photoreduce NADP from water when supplemented with the copper-containing electron transfer protein plastocyanin, ferredoxin, and ferredoxin NADP-reductase.

A preliminary account of some of the results in this paper has been published (20).

MATERIALS AND METHODS

The growth of maize plants (*Zea mays* var. DS 606A) in a greenhouse, the isolation of mesophyll chloroplasts and bundle sheath chloroplast fragments, and the assay of chlorophyll and photochemical activities were as previously described (1), except that the volume of assay mixtures was 0.75 ml. *Sorghum bicolor* var. Texas 610 was treated similarly. The ratio of chlorophyll *a* to chlorophyll *b* in isolated bundle sheath chloroplasts was about 6.0. Chloroplasts were isolated from the leaves of *Pisum sativum* L. by following the procedure used for maize mesophyll chloroplasts.

Plastocyanin was isolated from leaves of silver beet (*Beta vulgaris*) following the procedure of Katoh *et al.* (15). The concentration of plastocyanin was determined assuming 2 g atoms of Cu per mole of protein and an extinction coefficient of 4.9×10^6 cm^2 per g atom Cu (15). Ferredoxin NADP-reductase was purified from extracts of spinach (*Spinacia oleracea*) leaves and ferredoxin from *Anacystis nidulans*. The photo-oxidation or photoreduction of plastocyanin by isolated chloroplasts was recorded using an Aminco-Chance dual wavelength spectrophotometer (American Instrument Co.). In all experiments the light incident on the chloroplast suspensions was provided by a tungsten lamp filtered through a Corning 2-60 red cutoff filter and two Corning 1-69 heat filters. The light intensity was 12.2×10^4 ergs cm^{-2} sec^{-1}, and the temperature was 23 C.

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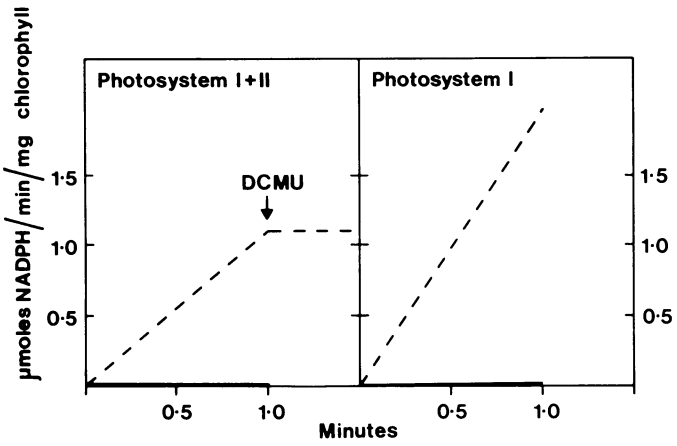

FIG. 1. The effect of plastocyanin on photosynthetic activities of maize bundle sheath chloroplasts. Photosystems I + II activity was measured as NADP photoreduction from water. Photosystem I activity was measured as NADP photoreduction in the presence of DCMU, DCIP, and ascorbate. Except for the use of plastocyanin, assays were made as described previously (1). ——: minus plastocyanin; - - - -: plus reduced plastocyanin (6.4 μM); arrow: addition of 2.5 μM DCMU.

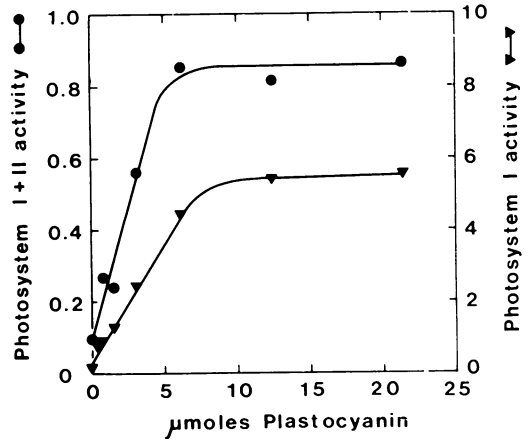

FIG. 2. The effect of plastocyanin concentration on photosynthetic activities in maize bundle sheath chloroplasts. Assays were as given in legend to Figure 1.

RESULTS

NADP Photoreduction by Agranal Chloroplasts. As described previously (4), washed agranal chloroplast fragments isolated by the method of Woo *et al.* (25) from the bundle sheath cells of maize leaves do not photoreduce NADP from water, and the photoreduction of NADP in the presence of ascorbate, DCIP,[2] and DCMU is low compared with isolated maize mesophyll chloroplasts (4). The addition of a crude centrifuged extract of maize leaves or an extract which had been concentrated by precipitation of protein with ammonium sulfate and then dialyzed gave only slight or insignificant increases in activity. However, the addition of plastocyanin to illuminated suspensions of bundle sheath chloroplast fragments resulted in photoreduction of NADP from water. The addition of ferredoxin was essential for activity under all conditions with both mesophyll and bundle sheath chloroplasts.

[2] Abbreviation: DCIP: 2,6-dichlorophenolindophenol.

Figure 1 shows the rates of NADP photoreduction with bundle sheath chloroplasts in the presence and absence of plastocyanin. It was difficult to detect a rate of NADP photoreduction from water (photosystems I + II) in the absence of the plastocyanin. Reduction of NADP in the presence of plastocyanin was light dependent and was inhibited by DCMU, showing that photosystem II is involved in the reduction. The addition of ascorbate and DCIP to the DCMU-inhibited chloroplasts produced a small but measurable photoreduction of NADP (photosystem I), but the activity was greatly stimulated by addition of plastocyanin.

Figure 2 shows NADP photoreduction at various concentrations of plastocyanin. Photoreduction of NADP from water was saturated at a plastocyanin concentration of about 6 μM, and photosystem I activity was saturated at a slightly higher concentration.

The effect of oxidized and reduced plastocyanin on NADP photoreduction from water is shown in Figure 3. Photoreduction of NADP began immediately in the presence of reduced plastocyanin. When oxidized plastocyanin was added, there was a lag of about 15 sec before photoreduction of NADP commenced, and a steady rate of reduction was reached after about 0.5 min.

NADP Photoreduction by Granal Chloroplasts. Figure 4 compares the activities of maize mesophyll and pea chloroplasts for photoreduction of NADP in the presence and absence of plastocyanin. The rate of NADP photoreduction by maize mesophyll chloroplasts was increased by plastocyanin, but the effect was variable, and with pea chloroplasts the increase in activity on the addition of plastocyanin was small. In the presence of plastocyanin the activities of maize bundle sheath chloroplasts, maize mesophyll chloroplasts, and pea chloroplasts were similar. Plastocyanin also increased photosystem I activity in maize mesophyll chloroplasts and increased slightly the activity in pea chloroplasts. Photosystem I activity of maize bundle sheath chloroplasts was two to three times higher than that of maize mesophyll or pea chloroplasts.

Table I shows that the requirement of added plastocyanin for NADP photoreduction by bundle sheath chloroplasts is not a peculiarity of maize. The two types of chloroplast isolated from *Sorghum* leaves exhibited the same differences.

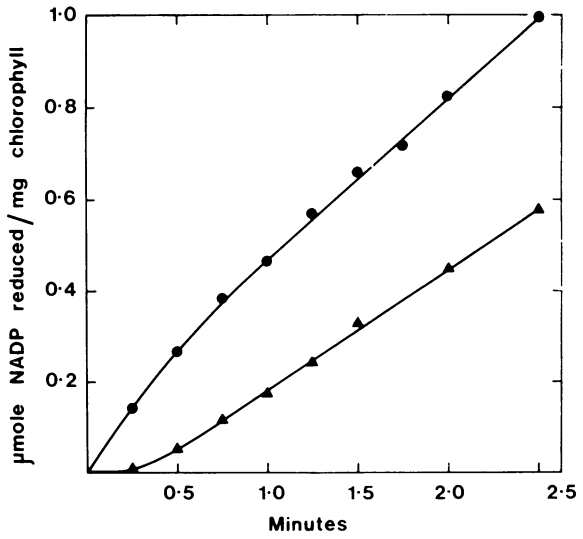

FIG. 3. NADP photoreduction from water by maize bundle sheath chloroplasts in the presence of plastocyanin. Assays were as given in Figure 1. ●——●: reduced plastocyanin present (3.2 μM); ▲——▲: oxidized plastocyanin present (3.2 μM).

Photoreduction and Photo-oxidation of Plastocyanin by Maize Chloroplasts. Figure 5 shows that when a mixture of 70% oxidized plastocyanin and maize bundle sheath chloroplasts is illuminated, the plastocyanin is reduced. Reduction is completely inhibited by 2.5 μM DCMU. The addition of an acceptor for photosystem I, such as methyl viologen, to the illuminated reaction mixture resulted in photo-oxidation of the plastocyanin. This reaction proceeded until the plastocyanin was approximately 50% oxidized. Elimination of the completing photoreduction by addition of DCMU resulted in further photo-oxidation until the plastocyanin was about 70% oxidized. In the absence of methyl viologen a slower photo-oxidation of the reduced plastocyanin ensued if DCMU was added to the illuminated reaction mixture. Photo-oxidation of reduced plastocyanin also occurred in the presence of ferredoxin and NADP (20), but the rate was lower than with methyl viologen.

Figure 6 shows an experiment with maize mesophyll chloroplasts similar to that described in Figure 5. Plastocyanin was again reduced upon illumination, but the addition of methyl viologen or DCMU plus methyl viologen (or DCMU alone) failed to promote photo-oxidation of plastocyanin.

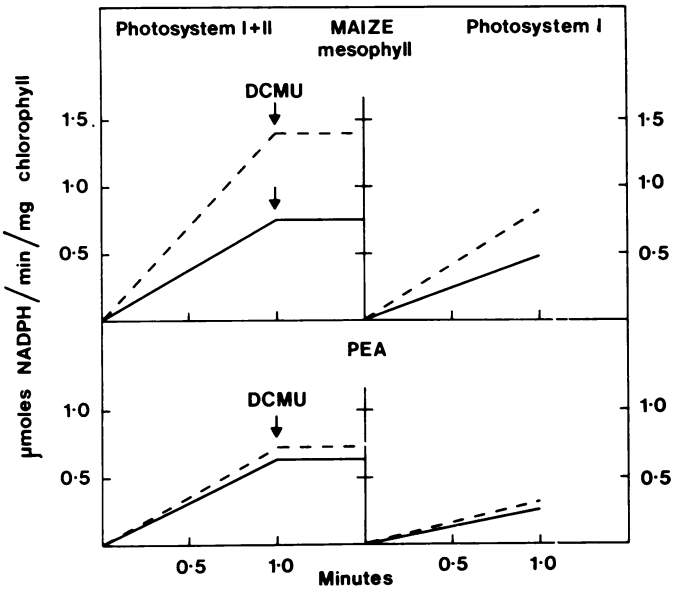

FIG. 4. The effect of plastocyanin on photosynthetic activities in maize mesophyll and pea chloroplasts. Assays were as given in legend to Figure 1.

Table I. *Photoreduction of NADP in Bundle Sheath and Mesophyll Chloroplasts from Sorghum*

Chloroplasts	Plastocyanin[1]	Photoreduction of NADP from Water[2]	Photosystem I Activity[3]
		μmoles NADPH/min·mg chlorophyll	
Bundle sheath	−	0	0
	+	0.89	1.31
Mesophyll	−	0.42	0.34
	+	1.03	0.55

[1] Where indicated, reduced plastocyanin (6.4 μM) was present.

[2] Reaction mixture contained sorbitol, 300 mM; phosphate buffer, pH 7.4, 10 mM; $MgCl_2$, 1 mM; NADP, 0.67 mM; ferredoxin, 3.3 μM; and chloroplasts, equal to 4.6 μg chlorophyll per ml.

[3] Reaction mixture contained the same mixture as above[2] with the addition of DCMU, 1.25 μM; DCIP, 67 μM; and sodium ascorbate, 2.5 mM.

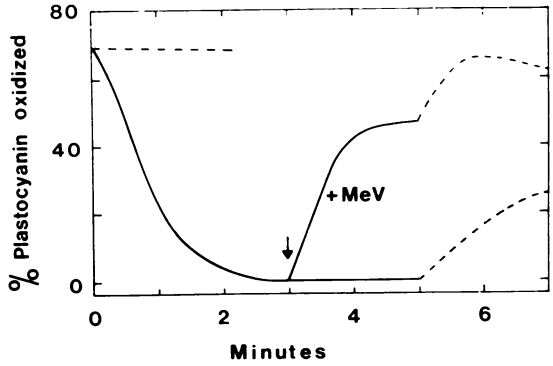

FIG. 5. Photoreduction and oxidation of plastocyanin by maize bundle sheath chloroplasts. The plastocyanin changes were recorded with measuring wavelength at 590 nm and reference wavelength at 570 nm. All changes shown are with illuminated samples. The reaction mixture contained plastocyanin (70% oxidized), 4 μM; sorbitol, 300 mM; potassium phosphate, pH 7.4, 10 mM; $MgCl_2$, 1 mM; and chloroplasts (4.6 μg chlorophyll/ml). Arrow: addition of methyl viologen (MeV) to give 1 mM; dashed lines: DCMU (2.5 μM) was present.

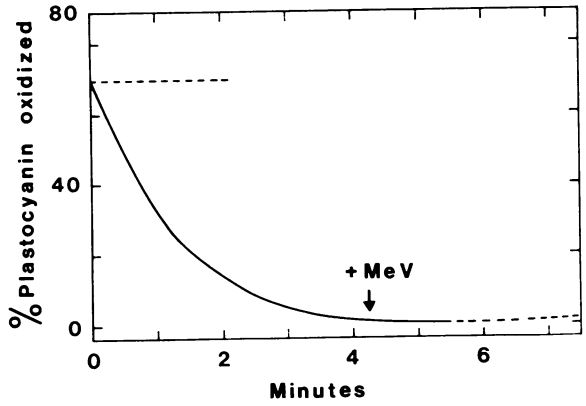

FIG. 6. Photoreduction of plastocyanin by maize mesophyll chloroplasts. Conditions were as described in Figure 5.

The Effect of Ferredoxin NADP-Reductase on NADP Photoreduction by Bundle Sheath Chloroplasts. The rates of NADP reduction by bundle sheath chloroplasts could be further stimulated by the addition of partially purified ferredoxin reductase. Under such conditions, rates of NADP photoreduction from water greater than 1.0 μmole/min·mg chlorophyll could be consistently obtained. The requirement of bundle sheath chloroplasts for plastocyanin was reduced but not eliminated by isolating the chloroplasts by a modified procedure (J. M. Anderson and N. K. Boardman, private communication), in which mercaptoethanol was replaced by 10 mM dithiothreitol and the leaves were prechilled before cutting. Maximal activity was still dependent, however, on the addition of ferredoxin reductase. Photosystem I activity of bundle sheath chloroplasts isolated by this procedure was about 6 μmoles NADP reduced/min·mg chlorophyll in the presence of plastocyanin and saturating amounts of ferredoxin reductase. In contrast, mesophyll chloroplasts prepared by the modified procedure showed photosystem I rates of 1.0 to 1.5 μmoles NADP reduced/min·mg chlorophyll, and the activity was not stimulated by plastocyanin. Ferredoxin NADP-reductase did not stimulate the activity of mesophyll chloroplasts prepared by either procedure.

DISCUSSION

The role of plastocyanin in photosynthetic electron transfer is generally accepted as that of an electron carrier situated between photosystems II and I (8). Plastocyanin can be removed from granal chloroplasts by sonication or treatment with detergents. Treatment of spinach chloroplasts with digitonin (3, 10, 16, 21, 24) or Triton X-100 (12, 23) resulted in the production of particles with photosystem I activity that was greatly stimulated by plastocyanin. Extraction of chloroplasts with heptane resulted in particles with similar properties (10). Sonicated chloroplasts showed apparent complete (3, 22) or partial loss (10, 13, 18) of photosystem II activity and loss of photosystem I activity which could be restored by the addition of plastocyanin.

In sonicated chloroplasts which still retained some photosystem II activity, NADP photoreduction from water could be demonstrated after the addition of plastocyanin (10, 13, 18). Plastocyanin also restored this activity in chloroplasts from mutants of *Chlamydomonas reinhardi* which lack normal plastocyanin (11). Arnon *et al.* (2) have obtained a particle by treatment of spinach chloroplasts with detergents and by sonication that shows a plastocyanin requirement for photoreduction of NADP from water. The particle lacks functional cytochrome *f* and P_{700}, although measurements of photosystem I in the presence of plastocyanin were not reported.

The requirement for plastocyanin and other soluble protein components in NADP photoreduction by agranal bundle sheath chloroplast preparations is most likely a reflection of both the chloroplast ultrastructure and the method of chloroplast isolation. *Euglena gracilis* chloroplasts, which also lack discrete grana and instead contain sheets of two or three appressed lamellae, show little activity for NADP photoreduction after isolation, either from water or DCIP-ascorbate. These activities can be restored by the addition of the soluble *c*-type cytochrome (cytochrome$_{552}$) from chloroplasts of *E. gracilis* (14) or of plastocyanin. Similar results have been obtained with chloroplasts isolated from *Porphyridium cruentum* (unpublished experiments). These chloroplasts contain no appressed lamellae. Presumably in all of these cases there would be no requirement for the addition of soluble proteins for NADP photoreduction if the chloroplasts were isolated in a completely intact state. In our experiments the bundle sheath chloroplasts were prepared according to the method of Woo *et al.* (25) and then well washed before resuspension for use in experiments. These preparations showed little activity for NADP photoreduction and a low activity for photosystem I. Slight modifications to the isolation procedure, including replacing mercaptoethanol by dithiothreitol (see "Results") resulted in preparations showing a reduced requirement for plastocyanin. In contrast, the same preparation after 20 sec of sonication showed an absolute requirement for plastocyanin (unpublished experiments). Thus agranal chloroplasts when damaged appear to lose soluble components more easily than granal chloroplasts, and the relative amounts of different components lost vary with such factors as the method used for isolating the chloroplasts, the composition of the isolation medium, and the time lapsed since the chloroplasts were released from the cells. Preparations which show little activity for NADP photoreduction and appear not to be linked between photosystem II and photosystem I may, in fact, be partially linked if they develop during isolation a requirement for ferredoxin NADP-reductase as well as for plastocyanin. This would explain the ferredoxin-dependent photoreduction of cytochrome *c* shown by bundle sheath chloroplasts from *Sorghum* even though the same preparations reduced NADP from water at very low rates (4). The photoreduction of cytochrome *c* by maize bundle sheath chloroplasts which show a reduced requirement for plastocyanin in NADP reduction is also stimulated by ferredoxin.

The nature of the requirement by bundle sheath chloroplasts for ferredoxin NADP-reductase is still not fully understood, since the bundle sheath chloroplast preparations show almost as much activity, measured as NADPH-diaphorase, as do the mesophyll chloroplasts (1). The possibility of a requirement for other, as yet unknown, components present as contaminants in the protein preparations has not been entirely eliminated. Traces of NADPH-diaphorase activity were usually detectable in preparations of plastocyanin. The ferredoxin NADP-reductase was estimated to be 50% pure on the basis of the flavin-to-protein ratio, but contained no detectable plastocyanin. The highly purified ferredoxin from *A. nidulans* contained neither plastocyanin nor NADPH-diaphorase activity.

The ratio of photosystem I activity to NADP photoreduction from water in the presence of plastocyanin is higher in bundle sheath chloroplasts than in mesophyll chloroplasts (data from Figs. 1 and 4), which could indicate a relative enrichment of photosystem I activity in bundle sheath chloroplasts as opposed to mesophyll chloroplasts. Such a conclusion is supported by the fact that maize and other plants containing the C4 pathway of photosynthesis contain a higher P_{700}-to-total chlorophyll ratio and a higher chlorophyll *a*:*b* ratio, than plants containing the C3 pathway (7). In addition, the proportion of fluorescence emitted by photosystem I relative to photosystem II in bundle sheath chloroplasts of *Sorghum* is greater than that of mesophyll chloroplasts (25).

In the isolated bundle sheath chloroplasts, plastocyanin appears to function as a link between the two photosystems as shown by its reduction by photosystem II (DCMU sensitive) and oxidation by photosystem I (DCMU insensitive). Mesophyll chloroplasts did not photo-oxidize plastocyanin (Fig. 6). However, this activity can be demonstrated in detergent-treated granal chloroplasts (17, 19). Although it cannot be concluded from our results that plastocyanin acts the same way *in vivo* in bundle sheath chloroplasts as *in vitro*, the results clearly show that isolated bundle sheath chloroplasts from maize and *Sorghum* possess the potential for electron flow between the two systems. Previous studies of the photoreduction and oxidation of cytochrome *f* in intact bundle sheath cells suggested that electron flow between the two photosystems does in fact occur *in vivo* (6). We would suggest that the agranal chloroplasts of these cells can generate NADPH upon illumination for use in photosynthesis and that under suitable conditions it will be possible to isolate agranal bundle sheath chloroplasts fully linked for electron flow.

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