Effect of Light on Chemical Modification of Chloroplast Ferredoxin-NADP Reductase

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NESTOR CARRILLO, HÉCTOR A. LUCERO, AND RUBÉN H. VALLEJOS
Centro de Estudios Fotosintéticos y Bioquímicos, (CONICET, F. M. Lillo, U. N. Rosario), Suipacha 531 2000 Rosario Argentina

ABSTRACT

Chemical modification of spinach chloroplasts by phenylglyoxal and dansyl chloride resulted in inhibition of NADP photoreduction. The rate of inactivation was higher with both reagents when modification was carried out in the light with methyliologen or phenazine methosulfate present. Uncouplers prevent the effect of light. Electron transport from water to methyliologen was not affected by the modifiers.

The presence of 10 millimolar NADP completely protected the membrane-bound reductase against inactivation by phenylglyoxal. With lower concentrations, protection was higher in the light than in the dark. The apparent dissociation constants of the enzyme-substrate complex for NADP were 0.9 and 0.1 millimolar for the dark and light inactivation, respectively. Inactivation of NADP photoreduction by dansyl chloride was completely prevented by ferredoxin, but only partially by nucleotides.

The diaphorase activity was inhibited in chloroplasts modified by phenylglyoxal, but not when modified by dansyl chloride.

The results suggest that energizing thylakoid membranes by light induces a conformational change in membrane-bound ferredoxin-NADP reductase, and that the reductase is an allotopic enzyme.

Reduction of the final acceptor of the photosynthetic electron chain, NADP, is catalyzed by a flavoprotein, ferredoxin-NADP reductase, first described by Avron and Jagendorf (3). The enzyme, which is tightly bound to the thylakoid membrane presumably by ionic forces in a crevice below the membrane surface and very near coupling factor 1 (4, 16), has been solubilized (14) and crystallized (19); it catalyzes the electron transfer from ferredoxin to NADP in the presence of pyridine nucleotide, which is also essential for activity. Both the arginyl and lysyl residues are thought to be involved in binding pyridine nucleotides to the catalytic site. Further, a sulfhydryl group is also essential for the catalytic activity of the soluble reductase (27).

These modifications were carried out with the soluble enzyme. It was important to determine if the essential arginyl and lysyl residues of the reductase were still accessible when the enzyme was bound to the thylakoid membrane, and whether light energization of the latter would affect this accessibility.

We found that the rate of chemical modification by phenylglyoxal and dansyl chloride of the membrane-bound ferredoxin-NADP reductase was enhanced by light energization of the membrane. This enhancement was probably achieved through a light-induced conformational change of the membrane-bound reductase.

MATERIALS AND METHODS

Chloroplast Isolation and Modification. Chloroplasts were isolated from fresh market spinach leaves (Spinacia oleracea L.) or from plants grown in a greenhouse as described (20), except that leaves were homogenized in a medium containing 250 mm sucrose, 5 mm MgCl2, 30 mm Tricine-NaOH (pH 8), and finally suspended in the same medium. Total Chl was determined as described (25). Ferredoxin was obtained from spinach leaves as described (6).

Treatment of chloroplasts with phenylglyoxal or dansyl chloride was carried out in dark or light at 30 C in a medium (1 ml) containing the same buffer and chloroplasts (200 µg Chl/ml). The modification of chloroplasts was terminated by centrifugation for 1 min at 12,000g in an Eppendorf microcentrifuge. The pelleted chloroplasts were washed twice in the dark and suspended in the buffer described. In some experiments the modification of chloroplasts was stopped by dilution in the reaction medium for the enzymic assay.

Enzyme Assays. Photoreduction of NADP was determined at 25 C in a reaction medium (1 ml) containing 250 mm sucrose, 30 mm Tricine-NaOH (pH 8), 0.5 mm NADP, ferredoxin in excess, 5 mm NH4Cl, and chloroplasts (50 µg Chl). The reaction was started by illumination (20,000 lux) and stopped after 3 min by addition of 50 µl 2 N NaOH. The pH was finally adjusted to 8 by adding 300 µl of a mixture containing 300 mm Tris-HCl (pH 8) and acetic acid. The samples were centrifuged and after measuring the initial A at 340 nm, 3 µg of PMS2 was added to oxidize the NADPH formed. The A after addition of PMS was taken as background.

Noncyclic electron transport from water to MV was measured

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2 Abbreviations: PMS: phenazine methosulfate; MV: methyliologen; Kd: dissociation constants; CF: chloroplast coupling factor 1.
as O₂ uptake as described (1).

Diaphorase activity of spinach chloroplasts was measured with ferricyanide as electron acceptor in a reaction medium (1 ml) containing 250 mM sucrose, 20 mM Tris-HCl (pH 8), 5 mM MgCl₂, 3 mM glucose-6-P, 1.2 mM ferricyanide, 0.25 mM NADP, glucose-6-P dehydrogenase (1 unit), and chloroplasts (10 μg Chl). The Δ decrease at 420 nm was measured.

Chemicals. Nucleotides, dansyl chloride, Tris, Tricine, MV, phenylglyoxal, and PMS were obtained from Sigma. All of the other chemicals were of analytical grade.

RESULTS

Inactivation of NADP Photoreduction by Phenylglyoxal and Dansyl Chloride. Figure 1 shows that incubation of spinach chloroplasts in the dark with phenylglyoxal resulted in inhibition of NADP photoreduction activity.

The reaction order for the inhibition of NADP photoreduction was determined from a plot of log k’ (k’ = ln2/tₐ₀) against log of phenylglyoxal concentration as previously used (17). The slope was nearly 1 (Fig. 1, inset), suggesting that inactivation of the uncoupled electron transport (from H₂O to NADP) was the consequence of the modification of only one residue of arginine per active site.

Similarly, chemical modification of chloroplasts by dansyl chloride also resulted in inhibition of NADP photoreduction. The inactivation followed apparent first order kinetics and the reaction order for dansyl chloride was nearly 1 (data not shown, but see below).

Photosynthetic electron transport from water to MV was affected neither by phenylglyoxal nor by dansyl chloride (Table I), suggesting that the action of these two modifiers is on the ferredoxin-NADP reductase. This is in agreement with the modification of arginyl and lysyl residues observed with the soluble enzyme from spinach (26) and Bumilleropsis (5). Phenylglyoxal was previously shown (15) to inhibit coupled, but not uncoupled electron transport from water to MV in spinach chloroplasts, and to modify coupling factor 1 (23). We confirmed that under our experimental conditions, uncoupled electron transport from water to MV was not inhibited by phenylglyoxal.

Effect of Light. When modification of chloroplasts by dansyl chloride or phenylglyoxal was carried out in the light (in the presence of PMS) instead of in the dark, the inactivation was 4.8 and 2 times more rapid, respectively, as shown in Table II. The same effect of light was observed with cyclic (PMS-dependent) and noncyclic (from water to MV) electron transport, but was slower in the absence of both reagents (Table II, exp. 1, line 2). Uncouplers like NH₄Cl completely prevented the effect of light, suggesting that energizing thylakoid membrane was necessary.

Protection against Phenylglyoxal. Complete protection against inactivation of the reductase by phenylglyoxal was afforded by 10 mM NADP (Table III). Other nucleotides including ATP and ADP also protected while ferredoxin did not afford any protection at all (Table III). When the modification of the chloroplasts with phenylglyoxal was carried out in light (Fig. 2B) instead of in dark (Fig. 2A), the rate of inactivation increased and protection by low concentrations of NADP was higher. From the data of Figure 2 on the influence of NADP concentration on the pseudo first order constant for inactivation of the reductase by phenylglyoxal, the apparent Kd of the enzyme-substrate complex can be calculated according to Scrutton and Utter (17) from the following equation:

$$\frac{k_1}{k_0} = K_1 + K_2 \left(1 - \frac{k_2}{k_0} \right)$$

where k’, and k₀ are the pseudo-first order constants for the inactivation in the presence and absence of substrate, respectively; K₁ and K₂ are the second order constants for the inactivation of

<table>
<thead>
<tr>
<th>Addition during Preincubation</th>
<th>O₂ Evolution (μmol/mg Chl·h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>142</td>
</tr>
<tr>
<td>Phenylglyoxal, 25 mM</td>
<td>142</td>
</tr>
<tr>
<td>Phenylglyoxal, 75 mM</td>
<td>139</td>
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Table II. Light Effect on Chemical Modification of Ferredoxin NADP Reductase

Half-times of inactivation of chloroplast NADP photoreduction (t₀ₐ) by modifiers and in conditions stated were determined as described in text. NH₄Cl, when present, was 5 mM.

<table>
<thead>
<tr>
<th>Conditions during Preincubation</th>
<th>NADP⁺ Photoreduction t₀ₐ (min)</th>
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<tbody>
<tr>
<td>Exp. 1: Modification with 100 μM dansyl chloride</td>
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<tr>
<td>Dark</td>
<td>36</td>
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<tr>
<td>Light</td>
<td>12</td>
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<tr>
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<tr>
<td>Light, MV</td>
<td>7.5</td>
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<tr>
<td>Light, MV, NH₄Cl</td>
<td>36</td>
</tr>
<tr>
<td>Exp. 2: Modification with 25 mM phenylglyoxal</td>
<td></td>
</tr>
<tr>
<td>Dark</td>
<td>21</td>
</tr>
<tr>
<td>Light, PMS</td>
<td>11</td>
</tr>
<tr>
<td>Light, PMS, NH₄Cl</td>
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</tr>
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</table>
TABLE III. Protection by Nucleotides of Ferredoxin-NADP Reductase against Inactivation by Phenylglyoxal

Chloroplasts were modified with 25 mM phenylglyoxal for 60 min in dark with additions stated. Nucleotides were 1 mM unless otherwise stated. Ferredoxin was 35 µM. NADP photoreduction was determined as described in the text. Control value was 50 µmol NADPH/mg Chl-h.

<table>
<thead>
<tr>
<th>Additions during Modification</th>
<th>NADP Photoreduction % of control</th>
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<tr>
<td>None</td>
<td>5</td>
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<tr>
<td>NADPH</td>
<td>62</td>
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<tr>
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<tr>
<td>ATP</td>
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<td>ADP</td>
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</tr>
<tr>
<td>Ferredoxin</td>
<td>4</td>
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</tbody>
</table>

FIG. 2. Protection by NADP of chloroplast ferredoxin-NADP reductase against modification by phenylglyoxal in dark and in light. Numerals on the slopes indicate NADP concentration (mM) present during dark (closed symbols) or light (open symbols) preincubation of spinach chloroplasts with 25 mM phenylglyoxal for time stated. Other experimental conditions were as described in text.

The free enzyme (E) and the enzyme-substrate complex (ES), respectively; and (S) is the concentration of substrate used. When the ratio \( k'_2/k'_0 \) is plotted against

\[
1 - \left( \frac{k'_2}{k'_0} \right) \frac{1}{(S)}
\]

the ordinate intersection represents \( K_2/K_1 \); the ratio of the second order constants for the reaction of ES and E with the inhibitor and the slope is \( K_0 \). The data of Figure 2 were plotted as indicated (Fig. 3) and we found that: (a) either in light or in dark the \( K_2/K_1 \) ratio obtained is zero showing that ES complex does not react with phenylglyoxal; (b) from the slope, \( K_0 \) for NADP of 0.9 and 0.1 mM were obtained for the dark and light inactivation, respectively (Fig. 3).

Protection against Dansyl Chloride. Ferredoxin completely protects NADP photoreduction against modification by dansyl chloride both in the light and in the dark (Fig. 4) at variance with the total lack of protection shown against phenylglyoxal (Table III). NADP and 5 mM NADPH also afforded considerable protection (Fig. 4), although it was not complete and it was higher in the dark than in the light at variance with the protection afforded against inactivation by phenylglyoxal.

Nearly complete reversal of the inactivation of NADP photoreduction by dansyl chloride was obtained within 3 min after adding 2.5 mM dithioerythritol either in the dark or in the light.

Effects ofModifiers on the Diaphorase Activity of Chloroplasts. Chemical modification of the soluble reductase by phenylglyoxal and dansyl chloride inhibited the diaphorase and transhydrogenase.

**FIG. 3.** Calculation of the Kd for complex between NADP and chloroplast ferredoxin-NADP reductase. Data from Figure 2 were plotted as described in text. Experiments in dark (closed symbols); experiments in light (open symbols).

**FIG. 4.** Protection of NADP photoreduction against dansyl chloride. Modification of spinach chloroplasts in dark (closed symbols) or in light (open symbols) by 100 µM dansyl chloride was carried out as described in the text with the following additions: (●, ○): none; (△, △): 5 mM NADP; (□, ■): 5 mM NADPH; (▲, ◆): 35 µM ferredoxin.
ase activities of the enzyme (5, 26). To confirm that the inactivation of NADP photoreduction reported in this paper was localized on the reductase, the diaphorase activity of chloroplasts was measured after modification by 25 mM phenylglyoxal in the dark or in the light. The half-times of inactivation were 25 and 11 min, respectively, in very good agreement with the values reported for inactivation of NADP photoreduction (Table II, exp. 2). Treatment of chloroplasts with 50-250 µM dansyl chloride either in the dark or in the light at pH 8 or 8.8 did not affect the diaphorase activity. Thus, at variance with the results of Zanetti (26) with the soluble spinach reductase the diaphorase activity of membrane-bound reductase was insensitive to dansyl chloride in the same conditions that completely inactivated NADP photoreduction.

**DISCUSSION**

The essential arginine per active site found in spinach chloroplasts modified with phenylglyoxal is at the pyridine nucleotide-binding site of the ferredoxin-NADP reductase as suggested by the complete protection afforded against inhibition of NADP photoreduction and diaphorase activities. The presence in the membrane-bound reductase of an essential arginine is in agreement with the results found with the soluble enzyme (5).

The results obtained by modification of chloroplasts with dansyl chloride are more difficult to interpret. The lack of effect on the diaphorase activity suggests that the reductase is an allosteric enzyme since the essential lysine from the NADP-binding site (26) is inaccessible to dansyl chloride in the membrane-bound enzyme. Therefore, the inhibition of NADP photoreduction by dansyl chloride in chloroplasts either may not be caused by modification of the reductase, or the residue affected is on the reductase, but is different from the lysine derivative in the soluble enzyme (26). The protection afforded NADP photoreduction by ferredoxin and nucleotides, and the lack of effect of dansyl chloride on the electron transport from water to MV, strongly support the latter alternative. The complete protection afforded by ferredoxin suggests that the modified residue is on the ferredoxin-binding site in the reductase. The nature of this residue is not clear although reversal of the effect by dithioerythritol suggests that it may be a sulphydryl group.

Ryrie and Jagendorf (13) have shown that light induced a conformational change in membrane-bound CF1. Chemical modification of membrane-bound CF1 was later shown to be dependent on, or affected by, light. Thus, a light-dependent inhibition of photophosphorylation in spinach chloroplasts was observed by sulphydryl reagents such as N-ethylmaleimide (11), 2,2’-dithio-bis-(5-nitropyridine) (2), o-iodosobenzoate (21), o-phenylene-dimaleimide (24), and by inorganic sulfate (12) and permanganate (7). On the other hand, chemical modification of the membrane-bound coupling factor by arginine reagents was facilitated by light in *R. rubrum* chromatophores (22) and in spinach thylakoids (Viale, Andreo, and Vallesos, unpublished observations). The enhancement of the sensitivity of NADP photoreduction and diaphorase to modifiers and the change in the apparent Kₜ₄ for NADP brought about by illumination of chloroplasts, suggests that energizing the thylakoid membrane by light induces a conformational change in the membrane-bound ferredoxin-NADP reductase and that it may play a role in its regulation.

**LITERATURE CITED**

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