Dependence of Nitrite Reduction on Electron Transport in Chloroplasts

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ABSTRACT

Methyl viologen and phenazine methosulfate (photosystem I electron acceptors), 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU, electron-transport inhibitor), and methylamine (photophosphorylation uncoupler) were used to study the dependence of nitrite reduction on electron transport in chloroplasts. DCMU, methyl viologen, and phenazine methosulfate markedly inhibited, whereas methylamine stimulated NO$_3^-$ reduction in isolated, intact spinach (Spinacia oleracea L.) chloroplasts. The addition of DCMU to leaf sections of spinach and corn, (Zea mays L. var. XL81), incubated with NO$_3^-$, caused no inhibition of nitrate reduction but inhibited nitrite reduction leading to the accumulation of NO$_2^-$ in the light. The addition of methylamine to comparable leaf sections did not affect either nitrate or nitrite reduction.

We concluded that: (a) nitrite reduction is functionally associated with the electron transport arising from the light reactions of the chloroplast and this provides additional support for the localization of nitrite reductase in the chloroplast; (b) nitrite reduction is associated with photosystem I and ferredoxin is the most likely donor in leaf tissue; and (c) ATP is not involved directly in nitrite reduction. However, ATP synthesis, by regulating electron flow to photosystem I, can affect nitrite reduction in the light.

Most (7, 14, 21) but not all (9, 13) studies have indicated that NiR$^-$ in leaf tissue is localized in the chloroplasts. Recently, additional confirmation of the chloroplastic location has been provided (15, 17). These studies demonstrated that isolated, intact chloroplasts, without supplemental cofactors or enzymes, could reduce NO$_3^-$ when illuminated. The rates of NO$_3^-$ reduction were equivalent to in situ assimilation rates and the amounts of NO$_2^-$ reduced were stoichiometrically associated with production of $\alpha$-amino nitrogen.

Work with isolated NiR (14) and illuminated, broken chloroplast preparations supplemented with Fd, suggests that reduced Fd is the electron donor for NO$_3^-$ reduction in leaf tissue. Because nonchlorophyllous tissue can reduce NO$_3^-$, but apparently lacks Fd, and because of the inhibitor work of Trebst and Burba (22), questions about the role of Fd in NO$_3^-$ reduction in leaf tissue have been raised (4).

Klepper (12) has shown that photosynthetic herbicides, including o-phenanthroline, a compound known to interfere with electron transport in the chloroplasts (10), interfere with the assimilation of NO$_3^-$ in illuminated leaf sections provided with NO$_3^-$ as the electron source. Although this work suggests that reduced Fd is the most probable electron donor for NO$_3^-$ reduction, it is not conclusive. It does not rule out the possible participation of ATP as suggested by Kessler (11) and Bourne and Milfin (6). However, when Milfin (16) used leaf tissue instead of root tissue, he found that ATP did not appear to be associated with NO$_3^-$ reduction.

The objective of this study was to use isolated, intact chloroplasts and leaf sections to: (a) show that NO$_3^-$ reduction is functionally associated with the electron-transport system of the chloroplasts; (b) seek additional evidence that Fd is the electron donor for NO$_3^-$ reduction in leaves; and (c) evaluate the concept that ATP is not an obligatory metabolite in NO$_3^-$ reduction.

MATERIALS AND METHODS

Plant Material. Freshly harvested, young (not fully expanded) leaves of greenhouse-grown spinach (Spinacia oleracea L.) were used for the chloroplast preparations and leaf sections. Leaf sections were also obtained from the fourth leaf of 15-day-old corn (Zea mays L. var. XL81) seedlings cultured on vermiculite with full strength Hoagland solution.

Chloroplast Preparation. The procedures for chloroplast preparation were as described (15), with the following exceptions. Iodoascorbate was omitted from the grinding medium A as well as the resuspension medium B. After the homogenate was filtered to remove cell debris, the filtrate was centrifuged at 1500g for 5 min. The pellet was resuspended with 5 ml of medium B, and centrifuged as before. The pellet was again resuspended in 5 ml of medium B and used as a source of intact chloroplasts. Previous work (15) has shown that similar preparations contained approximately 50% intact (Class I) chloroplasts.

Nitrite Assay. A 2-ml aliquot of the chloroplast preparation was combined with 0.25 ml of 5 mM KNO$_3$ and H$_2$O plus additives (photosynthetic inhibitor, electron acceptors, or uncoupler) to make a final volume of 3.0 ml. The reaction mixtures were incubated in a water bath at 30 C with continuous shaking. Light was provided by a 500-W flood lamp which gave an incident energy of approximately 1.5 $\times$ 10$^5$ ergs cm$^{-2}$ sec$^{-1}$. Nitrite loss was followed by removing 0.2-ml aliquots of the reaction mixture at zero and subsequent time intervals. Nitrite
was assayed as previously described (15). Chlorophyll concentrations were determined as described by Arnon (1).

**Additives.** DCMU was selected as a typical, photosynthetic electron-transport inhibitor whose inhibition site is associated with photosystem II (5, 10, 19). PMS and MeV can act as electron acceptors at a site associated with the primary acceptor of photosystem I (10, 19). PMS facilitates cyclic photophosphorylation (2). MeA uncouples photophosphorylation and stimulates electron flow (3, 8). The effect of these additives on the NO\textsuperscript{−}\textsubscript{2} reduction by the intact chloroplasts permits conclusions to be drawn pertinent to the objectives. The major effects and presumed sites of action of these compounds are shown in Figure 1.

**In Vivo Nitrate Reductase Assay with Leaf Sections.** Leaves of both corn and spinach were cut into squares approximately 5 mm\(^2\), weighed (0.3 g), and placed in 5 ml of infiltration medium held in a 50-ml beaker. The infiltration medium was composed of 0.15 M phosphate buffer, pH 7.5, 0.15 M KNO\textsubscript{3}, 0.04% Neutronyx 600 (Onyx Cml., Jersey City, N.J.), 1% propanol for the control, and the same medium plus the desired amount and kind of additive, i.e., inhibitor. The beakers and contents were transferred to a vacuum desiccator and twice evacuated to 6 mm Hg for 1 min. The beakers were transferred then to either a dark or lighted water bath at 30 C with continuous agitation. The incident light energy was 1.5 \(\times\) 10\(^4\) ergs cm\(^{-2}\) sec\(^{-1}\). Nitrite accumulation was measured by removing aliquots (0.5 ml) at the start (zero time) and at the end of the incubation period (60 min). At the concentrations reported in this paper, MeA and DCMU did not affect the activities of either nitrate or nitrite reductases per se. PMS did not affect the activity of nitrite reductase per se. All experiments were repeated at least twice.

**RESULTS AND DISCUSSION**

**Nitrate Assimilation by Chloroplasts.** The time course of nitrate assimilation by illuminated chloroplasts over a 30-min period (Fig. 2) is similar to data reported previously (15). However, when DCMU was included in the reaction mixture, NO\textsuperscript{−}\textsubscript{2} assimilation was inhibited markedly (75% of control after 30 min). Although this is not a new observation (18) it shows conclusively that blocking the flow of electrons from photosystem II to photosystem I interferes with NO\textsuperscript{−}\textsubscript{2} reduction.

The inhibitory effect of DCMU is concentration-dependent (Table I), with 50% inhibition at \(10^{-5}\) M.

MeV and PMS, compounds that are considered to accept electrons from "X", the primary acceptor from P\textsubscript{700} (system I, 10, 19), were also effective in inhibiting NO\textsuperscript{−}\textsubscript{2} reduction in isolated chloroplasts (Table I). Zweig, et al. (23) have shown that the photosynthetic herbicide, diquat, (a bipyridilium analogue, with biochemical functions similar to MeV) added at \(3 \times 10^{-4}\) M, to chloroplast preparations, caused an 85% inhibition of NADPH production (cf. MeV inhibition of NO\textsuperscript{−}\textsubscript{2} reduction, Table I).

The data (Table I) show that nitrite reduction is associated with photosystem I and Fd is the most likely electron donor for the light reduction of nitrite. Moreover, it has been shown that Fd can provide electrons for NiR in vitro (14) and Trebst and Barba (22) found that sulfoDSDP inhibited the reduction of NADP and NO\textsuperscript{−}\textsubscript{2} in broken chloroplast preparations. The inhibitor did not inhibit NiR, per se. In contrast, the same workers (22) found that the treatment of illuminated *Chlorella* cells with \(10^{-5}\) M DSPD reduced CO\textsubscript{2} fixation by 50% but did not alter NO\textsuperscript{−}\textsubscript{2} reduction. From this it was deduced that reduced Fd was not the electron donor for NiR in vivo. However, it is possible that at this level of inhibition of CO\textsubscript{2} fixation, NO\textsuperscript{−}\textsubscript{2} reduction is not appreciably affected. Support for this latter possibility was obtained by treating illuminated leaf discs with increasing concentrations of DCMU and measuring CO\textsubscript{2} fixa-

**Fig. 1.** Schematic representation of photosynthetic electron transport in chloroplasts and the proposed localization for nitrite reduction. Approximate location of the sites of action of DCMU, methyl viologen (MeV), phenazine methosulfate (PMS), and methylamine (MeA), are shown.

**Fig. 2.** Time course of NO\textsuperscript{−}\textsubscript{2} reduction by illuminated chloroplasts with and without DCMU. Preincubation time: 5 min in darkness.

**Table 1.** Inhibitory Effects of DCMU, MeV, and PMS on Nitrite Reduction by Illuminated Chloroplasts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NO\textsuperscript{−}\textsubscript{2} Reduced</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\mu)mol hr(^{-1}) mg Chl(^{-1})</td>
<td>%</td>
</tr>
<tr>
<td>Control</td>
<td>3.28</td>
<td></td>
</tr>
<tr>
<td>DCMU (10(^{-7}) M)</td>
<td>3.08</td>
<td>6</td>
</tr>
<tr>
<td>DCMU (10(^{-5}) M)</td>
<td>1.62</td>
<td>54</td>
</tr>
<tr>
<td>DCMU (10(^{-3}) M)</td>
<td>0.66</td>
<td>80</td>
</tr>
<tr>
<td>Control</td>
<td>4.00</td>
<td></td>
</tr>
<tr>
<td>MeV (3 (\times) 10(^{-5}) M)</td>
<td>0.74</td>
<td>81</td>
</tr>
<tr>
<td>PMS (3 (\times) 10(^{-5}) M)</td>
<td>0.74</td>
<td>81</td>
</tr>
</tbody>
</table>
Table II. Nitrite Reduction in the Light by Isolated Chloroplasts and Leaf Sections from Spinach

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chloroplasts</th>
<th>Leaf Sections</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles NO(_2) reduced h(^{-1}) mg Chl(^{-2})</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.60 (100)(^1)</td>
<td>7.00 (100)</td>
</tr>
<tr>
<td>+ MeA (10(^{-2}) M)</td>
<td>6.12 (170)</td>
<td>6.72 (96)</td>
</tr>
</tbody>
</table>

\(^1 C_0\) of activity.

Table III. Effect of DCMU and MeA on Nitrite Accumulation in Corn and Spinach Leaf Sections Provided with KNO\(_3\)

Experimental period was 60 min.

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment</th>
<th>NO(_2) Accumulated</th>
<th>Light/Dark</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μmoles/hr g fresh wt</td>
<td>%</td>
</tr>
<tr>
<td>Spinach</td>
<td>Control</td>
<td>1.08 0.19</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>DCMU (10(^{-4}) M)</td>
<td>1.49 1.09</td>
<td>73</td>
</tr>
<tr>
<td>Corn</td>
<td>Control</td>
<td>2.07 0.55</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>DCMU (10(^{-4}) M)</td>
<td>1.92 1.50</td>
<td>78</td>
</tr>
<tr>
<td>Spinach</td>
<td>Control</td>
<td>2.18 0.30</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>MeA (5 × 10(^{-2}) M)</td>
<td>2.08 0.31</td>
<td>15</td>
</tr>
<tr>
<td>Corn</td>
<td>Control</td>
<td>2.07 0.55</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>MeA (5 × 10(^{-2}) M)</td>
<td>2.21 0.38</td>
<td>17</td>
</tr>
</tbody>
</table>

not appear that ATP serves as a direct energy source for NO\(_2\) reduction.

Nitrate and Nitrite Assimilation by Leaf Sections. The effects of DCMU and MeA on NO\(_2\) assimilation in leaf sections in both dark and light are presented in Table III.

In the dark, DCMU caused some increase in the accumulation of NO\(_2\) by the spinach tissue but was without effect with the corn tissue. Unless DCMU blocks the flow of electrons from NADPH to Fd, there is no apparent reason why it should enhance NO\(_2\) accumulation. However, the results with the spinach tissue were reproducible and Klepper (12) has observed similar effects with O-phenanthroline.

In the light, the effects of DCMU on nitrite accumulation by the leaf sections were similar for both species and consistent with the chloroplast results. We concluded that DCMU reduced assimilation of NO\(_2\) by interfering with the flow of electrons.

The results show that leaf tissue containing NO\(_2\) and treated with any compound that interferes with electron flow in chloroplasts, without affecting nitrate reduction, will accumulate NO\(_2\) as proposed by Klepper (12). The accumulation of NO\(_2\), even at the low rates observed (Table III) in the immediate vicinity of the chloroplasts should be injurious to the plant.

Treatment of leaf sections with MeA caused no change in the accumulation of NO\(_2\) over that of the control tissue, with the possible exception of the corn tissue, in the light (Table III). The low amounts of NO\(_2\) accumulated in control tissue, in the light, indicates that in vivo, the rate of electron flow is sufficient to cope with the rate of nitrite produced by nitrate reduction. The lack of accumulation of NO\(_2\) in the light in MeA-treated tissue shows that ATP is not required for nitrite reduction. This assumes that MeA causes comparable uncoupling effects in leaf sections as with isolated chloroplasts.

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LITERATURE CITED

ELECTRON FLOW AND NITRITE REDUCTION


