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Dependence of Nitrite Reduction on Electron Transport in Chloroplasts[1]

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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_2^- 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[3] 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_2^- when illuminated. The rates of NO_2^- reduction were equivalent to *in situ* assimilation rates and the amounts of NO_2^- reduced were stoichiometrically associated with production of α-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_2^- reduction in leaf tissue. Because nonchlorophyllous tissue can reduce NO_2^-, but apparently lacks Fd, and because of the inhibitor work of Trebst and Burba (22), questions about the role of Fd in NO_2^- 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_2^- in illuminated leaf sections provided with NO_3^-. Although this work suggests that reduced Fd is the most probable electron donor for NO_2^- reduction, it is not conclusive. It does not rule out the possible participation of ATP as suggested by Kessler (11) and Bourne and Miflin (6). However, when Miflin (16) used leaf tissue instead of root tissue, he found that ATP did not appear to be associated with NO_2^- reduction.

The objective of this study was to use isolated, intact chloroplasts and leaf sections to: (*a*) show that NO_2^- reduction is functionally associated with the electron-transport system of the chloroplasts; (*b*) seek additional evidence that Fd is the electron donor for NO_2^- reduction in leaves; and (*c*) evaluate the concept that ATP is not an obligatory metabolite in NO_2^- 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. Isoascorbate 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 1500*g* 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_2 and H_2O 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 1.5×10^5 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

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[3] Abbreviations: NiR: nitrite reductase; Fd: ferredoxin; MeV: methyl viologen; PMS: phenazine methosulfate; MeA: methylamine; DSPD: disalicylidenepropanediamine.

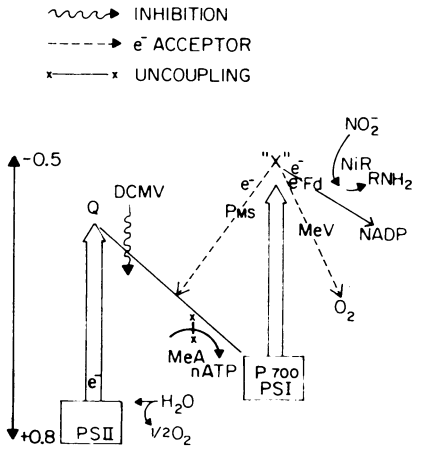

FIG. 1. Schematic representation of photosynthetic electron transport in chloroplasts and the proposed localization for nitrite reduction. Approximate location of the sites of action of DCMU, methyl viologen (MeV), phenazine methosulfate (PMS), and methylamine (MeA), are shown.

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_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_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 1.5×10^5 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

Nitrite Assimilation by Chloroplasts. The time course of nitrite 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_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_2^- reduction. The inhibitory effect of DCMU is concentration-dependent (Table I), with 50% inhibition at 10^{-6} M.

MeV and PMS, compounds that are considered to accept electrons from "X", the primary acceptor from P_{700} (system I, 10, 19), were also effective in inhibiting NO_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 3×10^{-5} M, to chloroplast preparations, caused an 85% inhibition of NADPH production (*cf.* MeV inhibition of NO_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 sulfoDSPD inhibited the reduction of NADP and NO_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^{-3} M DSPD reduced CO_2 fixation by 50% but did not alter NO_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_2 fixation, NO_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_2 fixa-

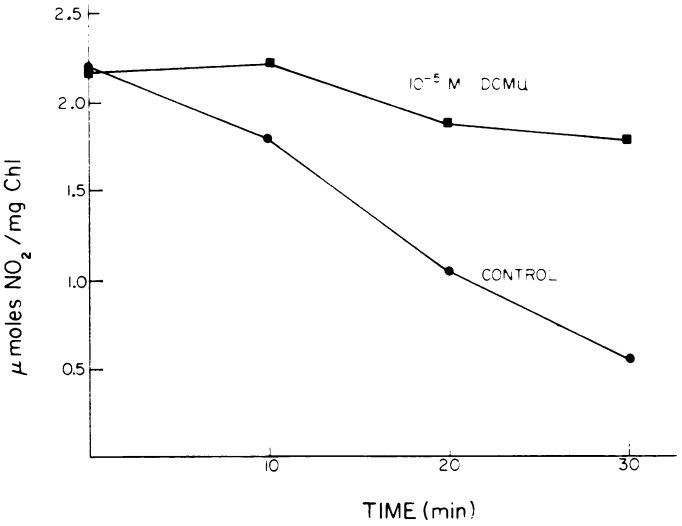

FIG. 2. Time course of NO_2^- reduction by illuminated chloroplasts with and without DCMU. Preincubation time: 5 min in darkness.

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

Experimental period was 30 min.

Treatment	NO_2^- Reduced	Inhibition
	μmoles hr⁻¹ mg Chl⁻¹	%
Control	3.28	
DCMU (10^{-7} M)	3.08	6
DCMU (10^{-6} M)	1.52	54
DCMU (10^{-5} M)	0.66	80
Control	4.00	
MeV (3×10^{-5} M)	0.74	81
PMS (3×10^{-5} M)	0.74	81

Table II. *Nitrite Reduction in the Light by Isolated Chloroplasts and Leaf Sections from Spinach*

Treatment	Chloroplasts	Leaf Sections
	μmoles NO_2^- reduced hr^{-1} mg Chl^{-1}	
Control	3.60 (100)[1]	7.00 (100)
+ MeA (10^{-2} M)	6.12 (170)	6.72 (96)

[1] % 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.

Species	Treatment	NO_2^- Accumulated		Light/Dark
		Dark	Light	
		μmoles/hr·g fresh wt		%
Spinach	Control	1.08	0.19	18
	DCMU (10^{-5} M)	1.49	1.09	73
Corn	Control	2.07	0.55	27
	DCMU (10^{-5} M)	1.92	1.50	78
Spinach	Control	2.18	0.30	14
	MeA (5×10^{-2} M)	2.08	0.31	15
Corn	Control	2.07	0.55	27
	MeA (5×10^{-2} M)	2.21	0.38	17

tion and NO_2^- reduction simultaneously (16). Inhibition of CO_2 fixation and NO_2^- reduction were 50% and 10% at 10^{-6} M DCMU, and 95% and 85% at 10^{-4} M DCMU, respectively. In leaf discs the process of CO_2 fixation appears more sensitive than NO_2^- reduction to these two inhibitors and does not rule out the possibility that reduced Fd is the *in vivo* electron donor for NiR. In contrast, Miflin (17) has shown with isolated chloroplasts that CO_2 fixation and NO_2^- reduction are inhibited equally by various concentrations of DCMU.

Experiments with MeA were conducted to determine whether ATP production resulting from electron flow in the chloroplasts was causally related to NO_2^- reduction. Data (Table II) show that the addition of MeA (5×10^{-2} M) to leaf sections of spinach infiltrated with NO_2^- did not affect the rate of NO_2^- reduction. However, the addition of MeA (10^{-2} M) to isolated chloroplasts enhanced NO_2^- reduction by 70% and made the rates comparable to the *in vivo* rates of leaf tissue. From these data, it is concluded that: (*a*) ATP is not an obligatory participant for NO_2^- reduction; (*b*) the uncoupling action of MeA caused an enhanced flow of electrons to the terminal electron acceptors of photosystem I and increased NO_2^- reduction; and (*c*) the phosphorylation site, by regulating electron flow, can serve as a control point for NO_2^- reduction. As indicated by Figure 1, the normal flow of electrons through the electron-transport system results in concomitant production of ATP and NO_2^- reduction. Under certain conditions ATP production could be accompanied by a stoichiometric reduction of NO_2^- (20), however, *in vivo* stoichiometry would not be expected necessarily.

Further evidence that ATP is not required for NO_2^- reduction *per se* was obtained by adding 10 μmoles of ATP to the reaction mixture. The chloroplast preparation receiving ATP reduced 87% as much NO_2^- as the control (3.84 *versus* 4.42 μmoles of NO_2^- hr^{-1} mg Chl^{-1}). From these data it does 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_3^- 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_3^- 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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