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ELECTRON TRANSPORT PATHWAYS IN SPINACH CHLOROPLASTS

REDUCTION OF THE PRIMARY ACCEPTOR OF PHOTOSYSTEM II BY REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE IN THE DARK

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Summary

Addition of NADPH to osmotically lysed spinach chloroplasts results in a reduction of the primary acceptor (Q) of Photosystem II. This reduction of Q reaches a maximum of 50% in chloroplasts maintained under weak illumination and requires added ferredoxin and Mg^{2+}. The reaction is inhibited by (i) an antibody to ferredoxin-$NADP^+$ reductase (EC 1.6.7.1), (ii) treatment of chloroplasts with *N*-ethylmaleimide in the presence of NADPH, (iii) disulfodisalicylidenepropanediamine, (iv) antimycin, and (v) acceptors of non-cyclic electron transport. Uncouplers of phosphorylation do not affect NADPH-driven reduction of Q.

It is proposed that electron flow from NADPH to Q may occur in the dark by a pathway utilising portions of the normal cyclic and non-cyclic electron carrier sequences. The possible in vivo role for such a pathway in redox poising of cyclic electron transport and hence in controlling the ATP/NADPH supply ratio is discussed.

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Abbreviations: sulfo-DSPD: disulfodisalicylidenepropanediamine; Tricine: *N*-Tris(hydroxymethyl)methylglycine; MalNEt: *N*-ethylmaleimide; PS I, Photosystem I; PS II, Photosystem II.

Introduction

In photosynthesis, electrons derived from water reduce $NADP^+$ by a series of intermediate reactions that includes two light-driven steps mediated by Photosystem I (PS I) and Photosystem II (PS II) [1]. Although this overall process is energetically unfavourable, there appears to be little reverse flow of electrons presumably because charge separation and stabilization steps in the PS I and PS II reaction centers are essentially irreversible in vivo [2].

On the other hand it is clear that redox reactions between certain intermediate carriers in the non-cyclic electron transport chain operate close to equilibrium so that, under certain conditions, electron flow can be driven in the reverse direction from that normally observed. For example, energization of the thylakoid membrane in the dark induces electron flow from cytochrome *f* to Q, the primary acceptor of Photosystem II [3,4]. This reaction involves plastoquinone as an intermediate and, although normally energetically unfavourable, can occur because a pH gradient across the membrane favours reduction of plastoquinone by cytochrome *f* [3,4].

At least part of the Q pool has a reported midpoint potential (E_m) of −50 to −90 mV at pH 8.0 [5,6]. Its reduction in the dark should therefore occur in the presence of NADPH (E_m (pH 8.0) ≈ −346 mV) provided that a pathway for this electron flow is present. In principle, some of the electron carriers involved in cyclic electron flow around PS I [1,7,8] might mediate a redox reaction between NADPH and Q allowing equilibrium to be established. Such a pathway has not yet been convincingly demonstrated but there are indications that it is present. Arnon and Chain [8] showed that NADPH stimulates cyclic photophosphorylation in isolated broken chloroplasts, which suggested that electrons from pyridine nucleotide can enter the cyclic pathway. These authors also suggested that some reduction of Q might occur, as judged by studies of absorption changes at 550 nm [8].

In this paper, reduction of Q by NADPH is demonstrated to occur in the dark. The electron flow pathway has been partially characterized and is shown to include portions of the normal cyclic and non-cyclic carrier sequences.

Materials and Methods

Intact chloroplasts were isolated from chamber-grown spinach as previously described [9] but with the modification introduced by Nakatani and Barber [10]. Intact chloroplasts were osmotically lysed in the cuvette, prior to measurement, by suspension in 10 mM $MgCl_2$ followed by addition of an equal volume of a medium containing 0.7 M sorbitol, 0.5 mM phosphate and 100 mM *N*-Tris(hydroxymethyl)methylglycine (Tricine) brought to pH 8.1 with KOH/NaOH. Occasionally a sorbitol-free medium consisting of 10 mM phosphate, 5 mM $MgCl_2$, 10 mM NaCl and 50 mM Tricine/NaOH was used for O_2 evolution measurements. Results were similar using either medium. Chloroplast concentrations were approximately 10 and 20 μg chlorophyll/ml for fluorescence and O_2 evolution measurements, respectively.

Chlorophyll *a* fluorescence excited by a weak modulated measuring beam (intensity, approx. 60 mW/m^2) was detected as previously described [11].

Additions to the cuvette (reaction volume, 2 ml) were made using a microsyringe. This procedure gave sufficiently rapid mixing whilst producing minimal mixing artifacts.

Oxygen evolution was measured by a Clark-type oxygen electrode as previously described [9].

Antiserum to ferredoxin-$NADP^+$ reductase was originally raised in rabbits by Dr. R. Berzborn at Cornell University, Ithaca, NY. Sera were stored in lyophilised form and were stable for several years. Sera were reconstituted in 0.1 M NaCl to give solutions approximately 10 mg protein/ml as determined by Lowry assay [12]. Undissolved particulate material was removed by centrifugation.

Disulfodisalicylidenepropanediamine (sulfo-DSPD) was a generous gift of Dr. A. Trebst, Ruhr Universitat, Bochum. Ferredoxin (type III) and NADPH (type III) were obtained from Sigma Co., St. Louis, MO.

Results

Illumination of osmotically lysed chloroplasts with a weak measuring beam (approx. 60 mW/m^2) excites a relatively low initial yield of chlorophyll *a* fluorescence (Fig. 1a). The low intensity of emission is due to the fact that Q, the primary acceptor of PS II is mainly oxidized. The PS II reaction centers are therefore open and able to trap excitation energy delivered from the bulk PS II chlorophyll. Fig. 1a shows that addition of NADPH causes a doubling in the yield of chlorophyll fluorescence. This NADPH-dependent fluorescence rise requires both ferredoxin and Mg^{2+}; if either of these cofactors is omitted, little fluorescence increase is seen on adding NADPH but an increase in emission will occur on adding the missing cofactor (ferredoxin, Fig. 1b or $MgCl_2$ Fig. 1c). In most experiments, the increase upon adding ferredoxin is slightly less pronounced than that observed on adding NADPH (compare Fig. 1a and b).

Fig. 2 shows the ferredoxin requirement of the NADPH-induced fluorescence rise. Saturation of both the final extent of fluorescence increase and the rate of the fluorescence rise ($t_{0.5}$) occurs at approximately 4—8 μM ferredoxin when the NADPH level is 0.25 mM. At higher levels of NADPH (1—2 mM), less ferredoxin is required and a small NADPH-dependent fluorescence increase is sometimes seen in the absence of added ferredoxin (results not shown). The ferredoxin requirement is similar to that observed for $NADP^+$ photoreduction (Ref. 13 and Table I). Again in the absence of added ferredoxin, some $NADP^+$ photoreduction can be observed, presumably catalysed by residual or bound ferredoxin present in these chloroplast preparations (see Table I). There is a similar cation requirement for both the NADPH-driven fluorescence increase and for $NADP^+$ photoreduction (Ref. 14 and Table I). These results suggest that the fluorescence rise seen on adding NADPH to broken chloroplasts in the dark results from reduction of the primary acceptor of PS II via a pathway that includes part of the non-cyclic electron transport chain. The cation requirement also incorporates the well-known stimulation of variable fluorescence yield seen mainly when PS II traps are closed [15].

The percentage of PS II traps that becomes closed may be calculated, assuming that all the fluorescence rise results from reduction of Q, by use of Eqn. 1

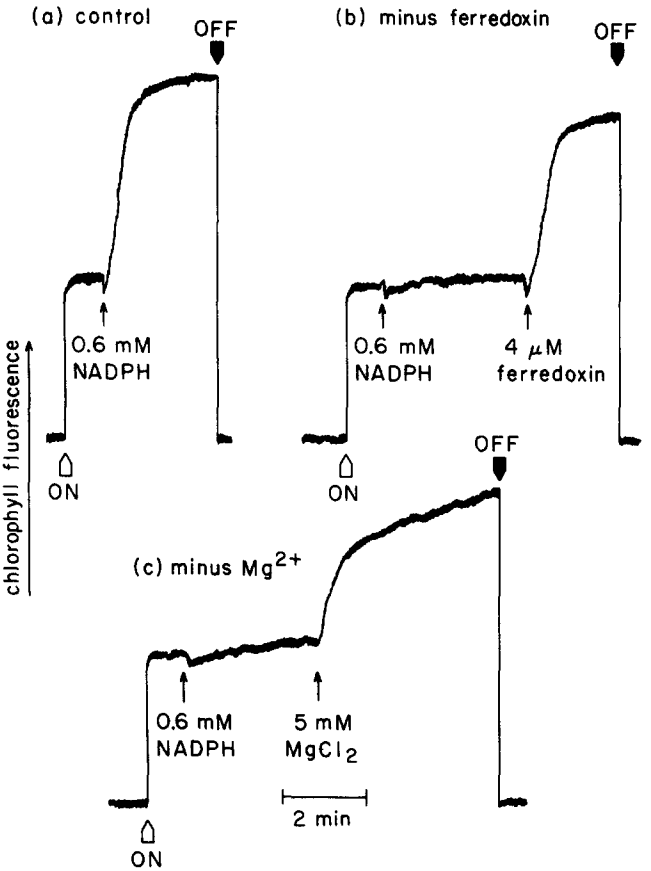

Fig. 1. Effect of NADPH addition on chlorophyll fluorescence emission in broken chloroplasts. The medium contained 0.35 M sorbitol, 0.25 mM phosphate, 4 μM ferredoxin, 5 mM $MgCl_2$, 50 mM Tricine, pH 8.1, and lysed chloroplasts, 9 μg chlorophyll/ml. NADPH was added by injection to a final concentration of 0.6 mM. 'On' and 'off' refer to switching the measuring beam.

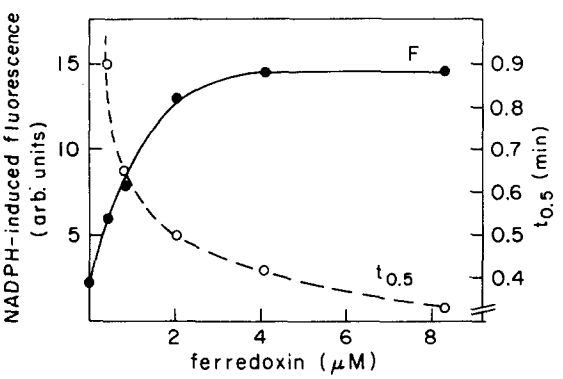

Fig. 2. Dependence of NADPH-induced fluorescence rise on added ferredoxin. Experiments were performed as shown in Fig. 1, except 0.25 mM NADPH was used. This level of NADPH was saturating for the highest ferredoxin concentration shown.

TABLE I

EFFECT OF VARIOUS COFACTORS ON LIGHT-DEPENDENT O_2 EVOLUTION AND NADPH-DEPENDENT FLUORESCENCE RISE IN BROKEN CHLOROPLASTS

For O_2 evolution, the medium contained 3.3 mM NH_4Cl, 4 μM ferredoxin, 1 mM $NADP^+$, 5 mM Mg^{2+}, and where indicated, 1 mM ferricyanide. Chloroplast concentration was 21 μg chlorophyll/ml. For fluorescence, the medium contained 0.6 mM NADPH in place of $NADP^+$ and, where indicated, 0.2 mM ferricyanide or a few crystals of solid sodium dithionite. Chloroplasts were added to a concentration of 10 μg chlorophyll/ml. The percentage of PS II traps closed was calculated from the relative fluorescence yield as discussed in the text.

Assay system	Rate of O_2 evolution (μmol/mg chlorophyll per h)	Chlorophyll fluorescence	
		Fluorescence yield (arbitrary units)	% PS II traps closed
Complete	118	47.5	48
Minus ferredoxin	28	22.0	11
Minus $NADP^+$ or NADPH	−19	21.0	9
Minus Mg^{2+}	64	24.0	—
Minus $NADP^+$ or NADPH, plus ferricyanide	124	16.0	0
Plus dithionite	—	104.0	100

originally derived by Joliot and Joliot [16] and recently discussed by Paillotin [17] and Hipkins [18],

$$\phi_{f,t} = \frac{(1-p)(1-q)}{1-p(1-q)} \tag{1}$$

where $(1-q)$ is the fraction of closed traps, and p is a complex function depending both on the probability of trapping excitation energy at the reaction center and on the probability of energy transfer between photosynthetic units [17,18]. Hipkins has recently estimated $p = 0.4$ in broken chloroplasts containing Mg^{2+} [18]. $\phi_{f,t}$ is the yield of variable fluorescence given by Eqn. 2

$$\phi_{f,t} = \frac{F_t - F_0}{F_m - F_0} \tag{2}$$

where F_t is the fluorescence seen at time t, F_0 is the minimum fluorescence seen when all traps are open and F_m is the maximum yield observed when all traps are closed.

F_0 and F_m were estimated from the data presented in Table I. In the presence of ferricyanide, fluorescence was assumed to be at F_0, whilst in the presence of dithionite which reduces Q [19], fluorescence was assumed to be at the F_m level. The percentage of closed traps was then calculated for the other conditions. In the absence of either ferredoxin or NADPH, the PS II traps were found to be approximately 9% closed, justifying the initial statement that under these weak illumination conditions (NADPH absent), fluorescence is almost at F_0. F_0 and F_m were not determined for the case where Mg^{2+} was omitted and where p will also be lower [18].

In the presence of all three cofactors, PS II traps were approximately 50% closed. This figure was consistently obtained (to within 10%) with different chloroplast preparations and could not be increased by adding a ten-fold higher

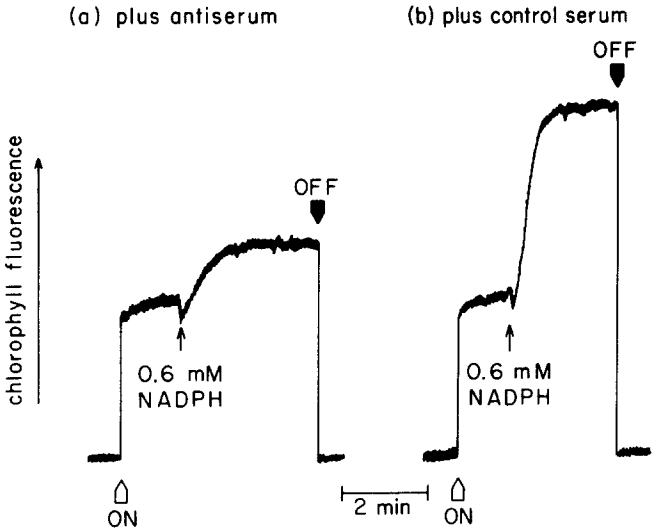

Fig. 3. Inhibition of NADPH-induced fluorescence rise by an antiserum to ferredoxin-NADP$^+$ reductase. Chloroplasts were preincubated for 3 min in the presence of (a) antiserum, 1.35 mg protein/ml, or (b) control serum, 1.5 mg protein/ml. Control serum had no effect on the NADPH-induced fluorescence rise.

concentration of NADPH. It is possible therefore that two pools of Q exist, only one of which can be reduced by NADPH.

It was of interest to establish the pathway of electron flow from NADPH to Q. NADPH is known to reduce ferredoxin by the activity of ferredoxin-NADP$^+$ reductase which is tightly bound to chloroplast membranes [20]. We therefore investigated the effects of inhibitors of this enzyme on NADPH-driven Q reduction.

Fig. 3 shows that an antiserum raised against pure reductase substantially (but not completely) inhibited the fluorescence changes. A control serum containing no reductase-specific antibodies had no effect. Table II shows that the antibody also inhibited light-dependent electron transport from H_2O to NADP$^+$ but not H_2O to ferricyanide. The results therefore show that both NADP$^+$

TABLE II

EFFECT OF FERREDOXIN-NADP$^+$ REDUCTASE ANTISERUM ON NON-CYCLIC ELECTRON TRANSPORT

The medium contained 10 mM NaCl, 5 mM $MgCl_2$, 10 mM phosphate, 1 mM NADP$^+$, 3 μM ferredoxin and 50 mM Tricine/NaOH, pH 8.1. Additions of 1 mM NH_4Cl and 1 mM ferricyanide were made to the same sample. Chloroplast concentration was 20 μg chlorophyll/ml.

Addition	Rate of oxygen evolution (μmol/mg chlorophyll per h)		
	—NH_4Cl	+NH_4Cl	+NH_4Cl + ferricyanide
None	61	96	90
150 μg/ml antiserum	53	61	82
300 μg/ml antiserum	45	45	—
600 μg/ml antiserum	27	27	113
600 μg/ml control serum	57	94	94

TABLE III

EFFECT OF *N*-ETHYLMALEIMIDE ON NADP$^+$ PHOTOREDUCTION AND NADPH-DEPENDENT FLUORESCENCE INCREASE IN SPINACH CHLOROPLASTS

At the time of assay, all samples contained 0.5 mM NADPH, 2.5 mM dithiothreitol, 5 mM *N*-ethylmaleimide and either 10 μg chlorophyll/ml (fluorescence) or 20 μg chlorophyll/ml (O_2 evolution) but the preincubation was varied as indicated in the first column. Fluorescence and O_2 evolution were otherwise measured as indicated in Tables I and II, respectively, except that ferredoxin addition was used to initiate the fluorescence increase.

Pretreatment (2 min)	Other additions before assay	Rate of NADP$^+$-dependent oxygen evolution (μmol/mg chlorophyll per h)		Fluorescence increase induced by ferredoxin	
				Extent (relative units)	$t_{0.5}$ (min)
		$-NH_4Cl$	$+NH_4Cl$		
NADPH, MalNEt, dithiothreitol	chloroplasts	77	132	24	0.6
Chloroplasts, NADPH, MalNEt	dithiothreitol	48	50	12	2.0
Chloroplasts, MalNEt	dithiothreitol NADPH	79	137	23	0.6

photoreduction and NADPH-driven Q reduction require ferredoxin-NADP$^+$ reductase activity.

The above conclusion was supported by studies utilising *N*-ethylmaleimide (MalNEt). This reagent reacts with free sulfhydryl groups to form a stable thioether derivative. Forti and Sturani [21] showed that MalNEt inhibited solubilized reductase providing the enzyme was reduced with NADPH. Table III shows that this reagent inhibits both NADPH-driven Q reduction and NADP$^+$ photoreduction providing that NADPH is present during preincubation. If NADPH is omitted, or if dithiothreitol-reacted MalNEt is used, no inhibition is observed. MalNEt did not inhibit ferricyanide photoreduction under any condition. Treatment of chloroplasts with MalNEt and NADPH thus appears to inhibit ferredoxin-NADP$^+$ reductase as is the case with the solubilized enzyme [21].

Sulfo-DSPD has been shown to inhibit ferredoxin-dependent reactions with both chloroplast and membrane-free systems [22,23]. Its site of inhibition has been placed before ferredoxin reduction in the electron transport sequence [22] or at ferredoxin itself [23]. Fig. 4 shows that sulfo-DSPD indeed inhibits non-cyclic electron transport to NADP$^+$, but not to ferricyanide, in accordance with earlier results [22,23]. Fig. 4 also shows the effects of sulfo-DSPD on fluorescence. Although this reagent does inhibit the fluorescence increase obtained after adding NADPH, there is also some decrease in emission in the absence of pyridine nucleotide. Clearly from Fig. 4, the relative increase in fluorescence induced by NADPH is inhibited by sulfo-DSPD. This might occur for two reasons: either sulfo-DSPD inhibits Q reduction by NADPH, or this reagent acts as a fluorescence quencher, specific for variable fluorescence. If the latter reason were predominant, it would be expected that the fluorescence increase induced by strong actinic light would by suppressed by sulfo-DSPD; this has not been observed (results not shown). It follows that sulfo-DSPD acts

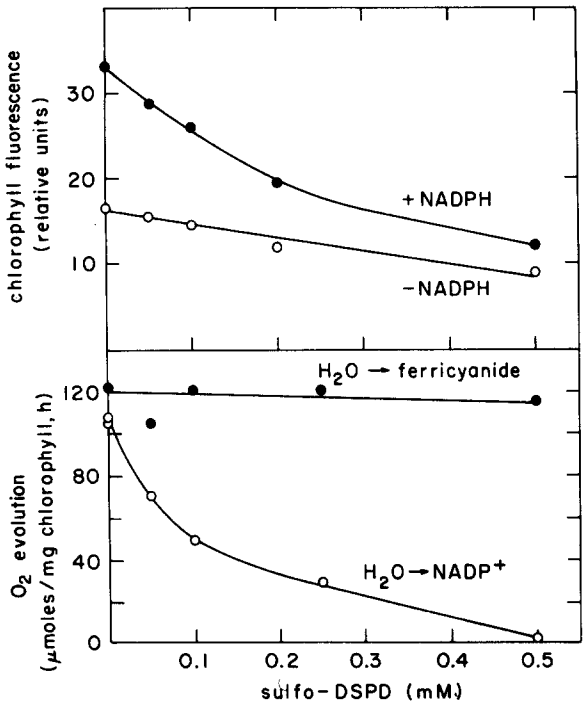

Fig. 4. Effect of sulfo-DSPD on chlorophyll fluorescence and O_2 evolution in lysed chloroplasts. Chlorophyll fluorescence was measured as indicated in Fig. 1 except that NADPH was omitted where indicated. O_2 evolution was measured as described in Table II except that all samples contained 1 mM NH_4Cl from the start.

mainly by inhibiting NADPH-dependent Q reduction. This effect is superimposed on an overall decrease in fluorescence that occurs in the presence of sulfo-DSPD.

Table IV lists the effects of antimycin, uncouplers and electron acceptors on NADPH-driven Q reduction. Antimycin, an inhibitor of cyclic electron transport in chloroplasts [8,9,11,24,25], is an effective inhibitor of NADPH-driven fluorescence changes, which suggests that electron flow to Q utilises a portion of the in vivo cyclic electron transport route. Freshly lysed broken chloroplasts carry out ferredoxin-mediated and antimycin-sensitive cyclic electron transport, though at reduced rates compared to those observed in the intact organelle (Mills, J.D. and Hind, G., unpublished observations).

The ionophores nigericin and valinomycin, either alone or in combination (Table IV) did not significantly affect either the rate or extent of the dark fluorescence rise; thus the rate of Q reduction by NADPH does not seem to be limited to concomitant development of an electrochemical proton gradient across the thylakoid.

Table IV also shows that class I electron acceptors of non-cyclic electron flow inhibit and slow down dark electron flow from NADPH to Q. Low concentrations of $NADP^+$ were quite inhibitory, presumably reflecting the fact that high ratios of NADPH/$NADP^+$ are required for significant reduction of ferredoxin. Ferricyanide (Table IV) and class III lipophilic electron acceptors

TABLE IV

EFFECT OF UNCOUPLERS, ANTIMYCIN AND ELECTRON ACCEPTORS ON NADPH-INDUCED FLUORESCENCE INCREASE

Conditions as given for Fig. 1 except the final concentration of NADPH was 0.25 mM.

Addition to control	NADPH-induced fluorescence increase (% control)	$t_{0.5}$ (min)
None	100	0.30
1 μM valinomycin + 1 μM nigericin	96	0.27
1 μM antimycin	34	0.70
$NADP^+$		
50 μM	64	0.70
200 μM	33	1.40
750 μM	26	1.90
Ferricyanide		
30 μM	78	0.85
75 μM	69	1.25
225 μM	41	2.65

(results not shown) also inhibited NADPH reduction of Q in the dark, presumably because electrons are removed from intermediate carriers before they reach Q.

Discussion

It has been shown in this paper that NADPH reduces part of the pool of primary acceptor in PS II, thereby closing PS II reaction centers in the dark. It is noteworthy that even in the presence of saturating amounts of NADPH and ferredoxin, only approximately 50% of Q becomes reduced in comparison to that reducible by dithionite. Several years ago, Cramer and Butler [5] presented redox titration curves for PS II fluorescence and showed two quenching processes with E_m at pH 8.0 of around −90 mV and −300 mV. These authors only assigned the more positive quenching component to Q. More recent data by Horton and Croze [6] suggest that the two quenching processes may reflect redox changes in two pools of Q, both being photoreducible by PS II. NADPH may be capable of reducing only the more positive of these quenchers (Q pools?) under the conditions reported here.

Fig. 5 represents a minimal pathway for the back flow of electrons from NADPH to Q based on our studies. It seems fairly certain that NADPH reduces ferredoxin via the activity of ferredoxin-$NADP^+$ reductase. This enzyme is inhibited by treatment with a specific antiserum or *N*-ethylmaleimide (in the presence of NADPH) and parallel inhibition of both $NADP^+$ reduction in the light, and NADPH-dependent Q reduction in the dark is observed.

As indicated in Fig. 5, electrons from ferredoxin probably are transferred to plastoquinone via a portion of the cyclic electron transport pathway that contains the antimycin inhibition site. Cytochrome b_6 may function in this region, since this electron carrier becomes partly reduced on addition of NADPH to the medium. Cytochrome b_6 reduction by NADPH is inhibited by antiserum to

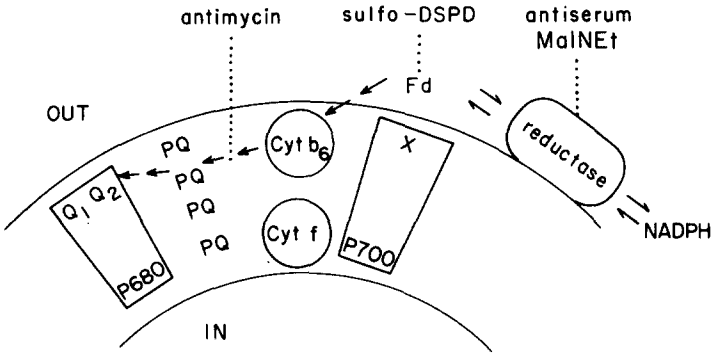

Fig. 5. Schematic pathway for electron flow between NADPH and Q in the dark. The proposed intermediate carriers are ferredoxin-$NADP^+$ reductase, ferredoxin (fd), cytochrome b_6 (cyt b_6) and plastoquinone (PQ).

reductase but not by antimycin (results not shown).

It is possible that the pathway involves a transmembrane proton transfer. Uncouplers did not stimulate the reaction but this may be explained if the overall rate-limiting step resides elsewhere. It should be noted that the rate of Q reduction by NADPH is slow. This may in part be due to an impairment of cyclic electron transport when intact chloroplasts are subjected to osmotic lysis (Mills, J.D. and Hind, G., unpublished observations). Substitution of a washed thylakoid preparation for osmotically lysed chloroplasts gave a reduction in rate and extent of NADPH-driven Q reduction of about 40%; EDTA-washed thylakoids showed further decreases (results not shown).

It is unclear whether back flow of electrons from NADPH to Q could compete kinetically with non-cyclic electron flow in vivo. The data in Table IV suggest that this would be unlikely unless the endogenous NADPH/$NADP^+$ ratio is high. Such a high ratio may occur in vivo during periods of high ATP demand, for example during the induction period of CO_2 fixation [26] or during concurrent carbon fixation and starch synthesis [27]. A limiting supply of ATP might be expected to result in considerable reduction of the pyridine nucleotide pool. Back flow of electrons to Q might then occur, inhibiting non-cyclic electron flow (by closing PS II traps) and stimulating cyclic photophosphorylation (as shown by Arnon and Chain [8]). In this way, the chloroplast could adjust the ratio of available ATP/NADPH by altering the balance of electron flux along the cyclic and non-cyclic pathways. Such a mechanism may in part account for recent observations that the activity of non-cyclic electron transport regulates cyclic photophosphorylation [11,28].

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