Biochimica et Biophysica Acta, 636 (1981) 234–243
Elsevier/North-Holland Biomedical Press

BBA 48082

THE INVOLVEMENT OF FERREDOXIN-NADP$^+$ REDUCTASE IN CYCLIC ELECTRON TRANSPORT IN CHLOROPLASTS

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(Received August 15th, 1980)
(Revised manuscript received February 20th, 1981)

Key words: Photosynthesis; Electron transport; Cyclic electron flow; Photosystem I; Ferredoxin-NADP$^+$ reductase; (Chloroplast)

The sites of action, in spinach thylakoid, of known inhibitors of electron transport at the reducing end of photosystem I have been more accurately located by parallel investigation of effects on three partial reactions: photoreduction (from water) of added NADP$^+$, photoreduction of added cytochrome *c*, and dark reduction of cytochrome *c* by added NADPH. Comparison with inhibitory effects on cyclic electron flow (registered by the slow phase of the electrochromic response during repetitive flash excitation) permitted assessment of the role of ferredoxin and ferredoxin-NADP$^+$ reductase (ferredoxin : NADP$^+$ oxidoreductase, EC 1.18.1.3) in the cyclic electron transport pathway around photosystem I. Disulfodisalicylidenepropane-1,1-diamine inhibited all the above partial reactions except the ferredoxin-dependent photoreduction of cytochrome *c*, thereby indicating its interference with the reductase or with complexation between reductase and ferredoxin. Studies with purified ferredoxin-NADP$^+$ reductase established it as the sensitive component. Cyclic flow is also sensitive to the above inhibitor and thus presumably involves the reductase. Supporting evidence for this came from studies of inhibition by substituted maleimides, which are inhibitors of electron transfer through the isolated reductase; these also inhibited the slow phase of the electrochromic response and all partial reactions except the photoreduction of cytochrome *c*. In contrast, an antiserum against the reductase affected only reactions involving NADP. The conclusion is drawn that the pathway of cyclic electron transport includes both ferredoxin and ferredoxin-NADP$^+$ reductase, but not the NADP-binding site on the reductase.

Introduction

Cyclic photophosphorylation catalyzed by ferredoxin was demonstrated first in broken chloroplasts to which ferredoxin was restored [1,2] and recently [3], in gently broken chloroplasts without supplementary ferredoxin. Its role in supplying some of the ATP needed during steady-state CO_2 fixation is well documented [4–7]; it is of particular importance during the induction phase of photosynthesis as a means of restoring pools of phosphorylated intermediates to the Calvin cycle [4,8]. Cyclic electron flow is sensitive to antimycin A [9–13] and DBMIB [2,14,15], and requires redox poising by PS II [2,6] and O_2 [7,11], or by added reductant [16]. Flash spectrophotometry reveals that antimycin A and DBMIB inhibit the turnover of cytochrome *b*-563 [15,17]; only the antimycin site seems to lie within the segment of the cyclic pathway that is not shared with linear electron flow between the photosystems [7,14,18].

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Abbreviations: Sulfo-DSPD, disulfodisalicylidenepropane-1,2-diamine; DBMIB, 2,5-dibromo-3-methyl-6-isopropylbenzoquinone; Mes, 4-morpholineethanesulfonic acid; MalNEt, *N*-ethylmaleimide; Tricine, *N*-tris(hydroxymethyl)methylglycine; DCIP, 2,6-dichlorophenolindophenol; PS, photosystem.

The question as to how electrons pass from PS I to cytochrome *b*-563 has been little explored. In linear electron transport, the reduction of $NADP^+$ by reduced ferredoxin is mediated by ferredoxin-$NADP^+$ reductase [19]. Recently, an antimycin-sensitive reduction by NADPH of the plastoquinone pool in unilluminated thylakoid membranes was also shown to involve this reductase [20,21]. In *Chlorella*, reoxidation of the reductase by an unidentified component of the cyclic pathway has been claimed [22] on the basis of a flash spectrophotometric study. A role in returning electrons from ferredoxin (loosely bound by ionic forces [3] to the thylakoid membrane outer surface) to buried membrane components such as cytochrome *b*-563 would be feasible for ferredoxin-$NADP^+$ reductase, which is more tightly bound [23] in a crevice shielded by the coupling factor complex [24]. Workers elsewhere, on the other hand, concluded from antibody studies [25] that the reductase was not involved in the cyclic pathway.

Selective inhibitors of ferredoxin-$NADP^+$ reductase are used here in conjunction with sensitive flash spectrophotometry to provide new evidence concerning the possible role of the reductase as an intermediate carrier in cyclic electron flow around PS I.

Materials and Methods

Intact spinach chloroplasts were isolated as previously described [7] except for substitution in the isolation medium of Mes for pyrophosphate. Chloroplasts were either assayed intact or were ruptured by shaking for 1 min in 1 mM Tricine (pH 8.2) followed by addition of an equal volume of double-strength reaction medium. The standard reaction medium contained 0.36 M sorbitol, 10 mM KCl, 5 mM $MgCl_2$, 500 U catalase/ml, 50 mM Tricine and 0.25 mM KH_2PO_4, pH 8.1. The chlorophyll concentration was 20 μg/ml unless stated otherwise. Initial intactness of the chloroplast outer envelope was routinely 70–85% as assayed by ferricyanide reduction [26]; the osmotic shock treatment resulted in total loss of intactness.

In intact or freshly broken chloroplasts, cytochrome *b*-563 turnover and cyclic generation of ΔpH and ATP [12,15,16] have been correlated with the slow rise ($P518_s$) of the electrochromic response ($P518$) [3] measured following flashes given at about 0.5 s intervals. Convincing evidence for the association of $P518_s$ with stoichiometric linear electron flow (from water to $NADP^+$) under comparable conditions is unavailable; indeed, acceptors such as methyl viologen which promote linear flow but inhibit cycling also inhibit $P518_s$ in repetitive flashes [8], while in intact chloroplasts, the partial closure of PS II by reduction of one of its acceptors enhances $P518_s$ [8]. Following a flash, $P518_s$ occurs concomitantly with the reduction of cytochrome *f* by a (special?) quinone [17]. It may be observed in linear electron flow when reduced quinone, reduced cytochrome *f* and an oxidized acceptor are available before each flash. Cyclic electron flow seems necessary for efficient regeneration of this state during repetitive flash illumination at the flash frequencies used here; under these conditions $P518_s$ serves as a reliable measure of that cyclic activity [3,8,12,15,16].

Flash-induced absorbance changes at 518 nm were retrieved and analyzed in a single-beam spectrophotometer linked to a PDP 11/34A computer as previously described [3,7,11,17]. The actinic flash was filtered through Schott RG 665 glass. Each sample was preilluminated by 40 flashes and the following 64 or 128 flash-induced transients were averaged. The flash frequency was 2.0 Hz.

O_2 evolution was measured with a Yellow Springs Instruments Clark-type electrode. Actinic light, provided by a tungsten source, passed through a Corning 4-76 filter and 10 cm of water; its intensity was 200 W/m^2 at the reaction vessel. Electron transfer from NADPH to cytochrome *c* in the dark was measured in a dual-wavelength spectrophotometer by observing the reduction of cytochrome *c* at 550 nm minus that at 540 nm. All reactions were run at 20°C.

Ferredoxin was obtained either from Sigma (type III, isolated from spinach and dissolved in 0.15 M Tris-HCl, pH 7.5) or from Miles-Yeda (isolated from Swiss chard, lyophilized); these were equally effective in the reactions described here. Cytochrome *c* (horse heart, type III) and spinach ferredoxin-$NADP^+$ reductase were obtained from Sigma. Antimycin A was obtained from Calbiochem. Antiserum raised in rabbit against ferredoxin-$NADP^+$

reductase, and control serum, were kindly given by Dr. R.E. McCarty, Cornell University, Ithaca. The lyophilized sera were dissolved in 0.15 M NaCl just before use.

Results

Fig. 1 displays the flash-induced electrochromic shift (*P*518) in intact chloroplasts, chloroplasts freshly ruptured in the absence of Mg^{2+} and similarly ruptured chloroplasts to which exogenous ferredoxin and Mg^{2+} were added. The fast rise of *P*518 was of equal extent in the three types of preparation but the slow rises ($P518_s$) were different: breakage in the absence of Mg^{2+} caused loss of $P518_s$ (Fig. 1b) which was restored to over 85% of that in intact preparations (Fig. 1a) by addition of ferredoxin and Mg^{2+} (Fig. 1c). Breakage in the presence of Mg^{2+} (data not shown) retained $P518_s$ and cyclic electron flow as shown previously [3]. Both the native and the restored $P518_s$ signals were sensitive to antimycin (Fig. 1) and to DBMIB [3]. The data confirm that $P518_s$ registers the activity of cyclic electron flow under the conditions of this study.

Four processes involving ferredoxin are compared in the following text; they are numbered and listed below for reference:

(1) $P518_s$, as an indicator of cyclic electron flow

(2) NADP photoreduction

$(H_2O \rightarrow \text{PS II} \rightarrow \text{PS I} \rightarrow \text{ferredoxin} \rightarrow \text{FNR} \rightarrow NADP^+)$

(3) cytochrome *c* photoreduction

$(H_2O \rightarrow \text{PS II} \rightarrow \text{PS I} \rightarrow \text{ferredoxin} \rightarrow \text{cytochrome } c)$ [27]

(4) cytochrome *c* dark reduction

$(\text{NADPH} \rightarrow \text{FNR} \rightarrow \text{ferredoxin} \rightarrow \text{cytochrome } c)$ [28]

Cyclic phosphorylation has been reported [2] to saturate with 10 μM ferredoxin (or 1 mol ferredoxin/33 mol chlorophyll) under aerobic conditions, and a similar value was claimed [2] for phosphorylation accompanying reduction of $NADP^+$. In agreement, more than 7 μM ferredoxin (7 mol/20 mol chlorophyll) was needed here to saturate processes 2 and 4 (Fig. 2b and d). However, as is clearly shown in Fig.

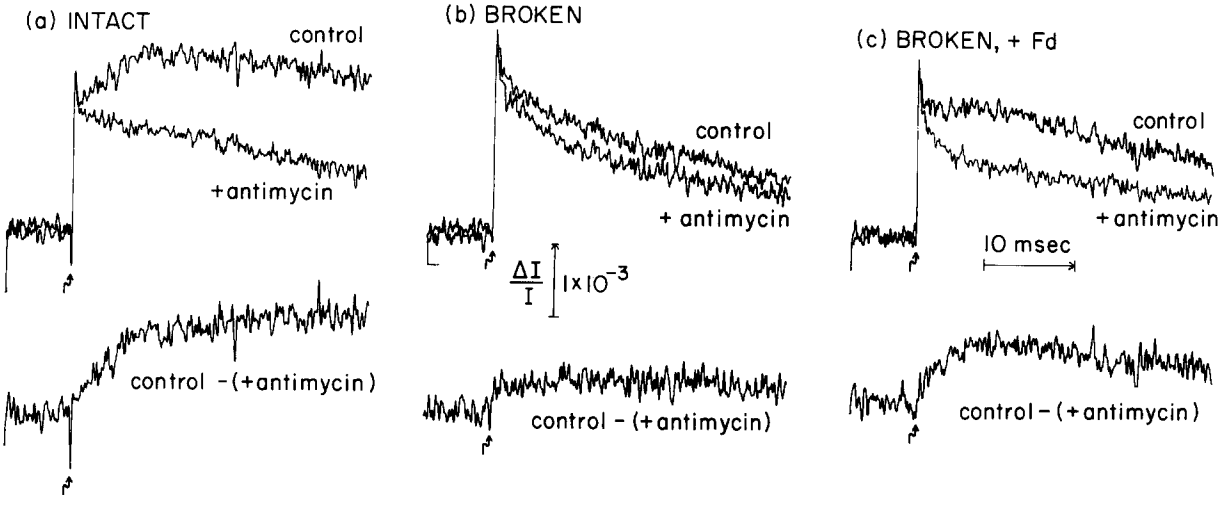

Fig. 1. Flash-induced absorbance change at 518 nm in intact and broken chloroplasts. Chloroplasts were assayed intact (a) or were osmotically shocked in the absence (b and c) of $MgCl_2$, as described in Materials and Methods. The final reaction mixture was the same in each sample and was as described in Materials and Methods, with inclusion of 1.8 μM nigericin. In c, 2.8 μM ferredoxin was added. Antimycin A was added to each sample after the first set of flashes and another set was then given. The concentration of antimycin A needed for full inhibition was higher for intact (27 μM in a) than for broken chloroplasts (13 μM in b and c), probably because of binding to stromal proteins [48].

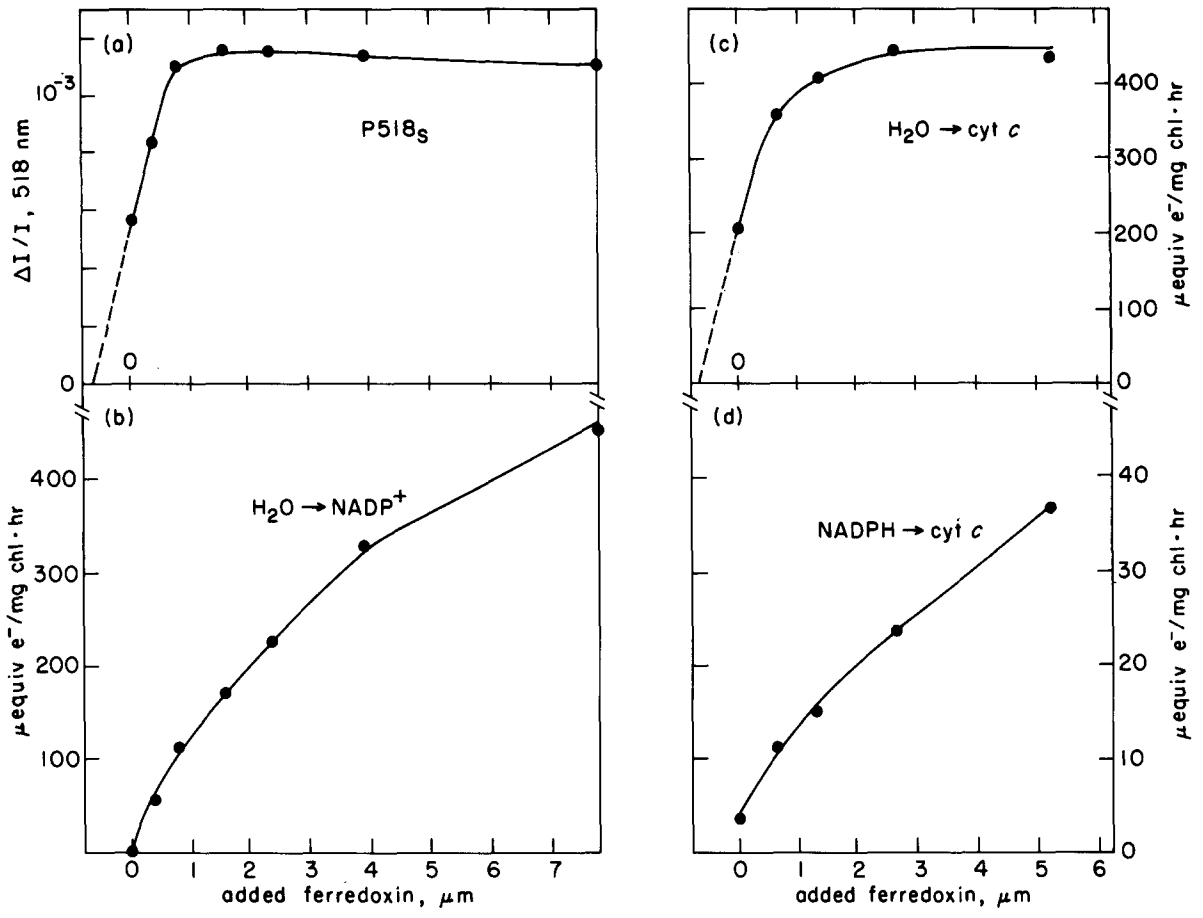

Fig. 2. Ferredoxin concentration dependences of the slow absorbance change at 518 nm and three different electron transport reactions. Intact chloroplasts (100 μg chlorophyll) were shocked in 2.5 ml of 1 mM Tricine, pH 8.2, by stirring on a vortex mixer for 1 min then 2.5 ml double-strength reaction mixture, 1500 U catalase, 1.4 μM nigericin and the indicated concentration of ferredoxin were added (final $[K^+]$ = 10 mM). Of this mixture, 1.5 ml were taken for absorbance and 3.0 ml for $NADP^+$ reduction assays. In the former assay, 64 flash-induced responses were averaged then 13 μM antimycin A was added and the process repeated; the difference between these sets is presented in a (cf. Fig. 1). In the latter, light-induced O_2 evolution (b) was determined after addition of 0.5 mM $NADP^+$. For traces c and d, intact chloroplasts (60 μg chlorophyll) were broken in 1.4 ml of 1 mM Tricine, pH 8.2, by shaking in a cuvette for 1 min; double-strength reaction mixture (1.5 ml), 1250 U catalase, 3.3 mM NH_4Cl, 0.5 mM cytochrome *c* and ferredoxin at the indicated concentration were then added. Photoreduction of cytochrome *c* was followed as O_2 evolution (c) and NADPH-driven dark reduction of cytochrome *c* was followed as the absorbance change at 550 nm after adding 0.67 mM NADPH (d). For other details see Materials and Methods.

2a and c, the ferredoxin concentration dependence of cyclic electron flow (process 1) was similar to that for photoreduction of externally added cytochrome *c* (process 3) but different from that of processes 2 and 4, which involve $NADP^+$/NADPH. Maximal activity was restored to processes 1 and 3 by 1–2 μM ferredoxin (1–2 mol/20 mol chlorophyll). Differences are also evident in the activities remaining after osmotic shock in the absence of added Mg^{2+}: processes 1 and 3 retain 25–45% of the ferredoxin-saturated rate whereas the reactions involving NADP have less than 10% residual activity. From extrapolation of the plots in Fig. 2a and c to zero activity, it is estimated that 0.5 mol ferredoxin/20 mol chlorophyll was still present after breakage (most probably bound to the thylakoid membrane, since the suspen-

sion was diluted 300-fold before assay).

Processes 2 and 4 have a common dependence on the $NADP^+$/NADPH couple and its binding site on the reductase [29–33], whereas the photoreduction of cytochrome *c* (process 3) may be independent of reductase [27] or may require it only as a ferredoxin-binding site on the membrane. The superficial resemblance between cyclic flow and cytochrome *c* photoreduction, seen in Fig. 2, was further examined by studies with selective inhibitors.

The effect of antiserum to the reductase, in the presence of added ferredoxin, is summarized in Table I. Photoreduction of $NADP^+$ and the dark reduction of cytochrome *c* by NADPH were clearly inhibited by the antireductase serum, whereas $P518_s$ and the photoreduction of cytochrome *c* were essentially unaffected; thus the results agree with those obtained from studies of ferredoxin concentration dependence (Fig. 2) in setting apart processes that involve pyridine nucleotide from those that do not.

Other inhibitors revealed a different pattern of sensitivity, however. Sulfo-DSPD, which does not enter the thylakoid membrane and is hence more selective than DSPD, was alleged to inhibit electron transport between PS I and ferredoxin [34] or at the ferredoxin site [18,35]. Its effect on the four ferredoxin-dependent electron transport processes

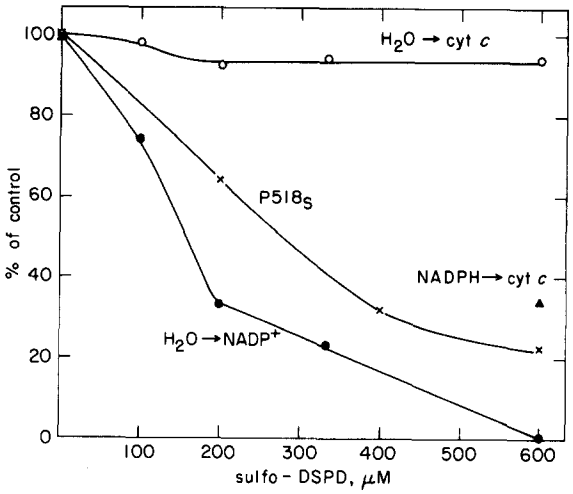

Fig. 3. Effect of sulfo-DSPD on various ferredoxin-dependent reactions. Chloroplasts were freshly shocked without $MgCl_2$ as described in Materials and Methods; after addition of double-strength reaction mixture, 1.7 μM ferredoxin, 1.4 μM nigericin and the indicated concentration of sulfo-DSPD were added in the order given. Electron transport from water to $NADP^+$ (●———●) or to cytochrome *c* (○———○) was measured as O_2 evolution with 0.5 mM $NADP^+$ or cytochrome added. Electron transport from NADPH to cytochrome *c* (▲———▲) was measured at 550 nm minus 540 nm in the dark following addition of 0.67 mM NADPH. Control rates were 513, 806 and 50 μequiv./mg chlorophyll per h for processes 2, 3 and 4, respectively. For other details see Materials and Methods.

TABLE I

EFFECT OF ANTISERUM AGAINST FERREDOXIN-$NADP^+$ REDUCTASE ON VARIOUS FERREDOXIN-DEPENDENT REACTIONS

$NADP^+$-dependent evolution of O_2 and flash-induced absorbance changes at 518 nm were measured in samples prepared as described for Fig. 2a and b, with the addition of 2.8 μM ferredoxin. Electron transport to cytochrome *c* from water or NADPH was measured as in Fig. 2c and d in the presence of 3.3 μM ferredoxin. Where shown, control serum or antiserum (0.5 mg protein/ml) was added after osmotic shock and incubated for 3 min before addition of double-strength reaction mixture. Initial rates of electron transport are given in μequiv./mg chlorophyll per h. The extent of the DBMIB-sensitive slow *P*518 change is given as $(\Delta I/I)(\times 10^{-3})$.

Addition	Process							
	1 ($P518_s$)		2 ($H_2O \rightarrow NADP^+$)		3 ($H_2O \rightarrow$ cytochrome *c*)		4 (NADPH → cytochrome *c*)	
	Extent	%	Rate	%	Rate	%	Rate	%
None	1.33	100	327	100	816	100	40.0	100
Control serum	1.27	95	308	94	884	108	–	–
Antiserum	1.22	92	53	16	916	112	3.8	10

is shown in Fig. 3. Photoreduction of $NADP^+$ (process 2) was inhibited by sulfo-DSPD (with 50% inhibition at 0.15 mM) as reported before [34]; however, photoreduction of cytochrome *c* (process 3) was scarcely affected by sulfo-DSPD at concentrations up to 0.6 mM. Since cytochrome *c* is directly reduced by ferredoxin [27] whereas $NADP^+$ reduction is mediated by the reductase, the above results show that sulfo-DSPD inhibits the reductase itself or electron transfer from ferredoxin to the reductase.

The site of inhibition was clarified by studies with isolated reductase. Table III shows that the purified enzyme is sensitive to 600 μM sulfo-DSPD whether catalyzing the electron flow from NADPH to dye in a ferredoxin-independent diaphorase reaction, or the much faster electron transport to cytochrome *c*. When the enzyme is not preincubated with the inhibitor (values in parentheses), the initial electron flow rate is only slightly depressed and full inhibition sets in during the first 30 s of assay, resulting in a continually declining rate; this result shows that sulfo-DSPD reacts slowly with the reductase and is not simply serving as a competing electron acceptor [35]. In view of this new evidence that sulfo-DSPD reacts directly with the reductase in the absence of ferredoxin, and the demonstrated failure of sulfo-DSPD to inhibit photoreduction of cytochrome *c* (Fig. 3), the inhibitory effect of sulfo-DSPD on $P518_s$ strongly indicates involvement of the reductase in cyclic electron flow.

Millimolar concentrations of MalNEt or micromolar concentrations of its lipophilic analog *N,N'-o*-phenylenedimaleimide are known to esterify active sulfhydryl groups of polypeptides that function on the thylakoid membrane exterior. Photophosphorylation is inhibited by specific interaction with a thiol group on the γ-subunit of CF_1 [36,37], while MalNEt inactivates the reduced form of isolated ferredoxin-$NADP^+$ reductase [38]. MalNEt is presumed to inhibit membrane-bound reductase similarly, for its inhibition of dark electron flow between NADPH and the reducing side of PS II [20] shows the characteristic protection by thiols and dependence on preincubation under reducing conditions. The effect of MalNEt or *N,N'-o*-phenylenedimaleimide was tested on processes 2–4 after preillumination in the presence of nigericin; under these conditions, the maleimides would be expected to react with reductase but not wth CF_1 as the reactive site of the latter is not exposed in the absence of a proton gradient [36]. The results (Table II) show that processes involving NADP are sensitive to MalNEt or

TABLE II

EFFECT OF MALEIMIDES ON FERREDOXIN-$NADP^+$ REDUCTASE IN FRESHLY BROKEN CHLOROPLASTS

Chloroplasts were osmotically shocked in 1 mM Tricine, pH 8.1, then an equal volume of 0.72 M sorbitol was added. NADPH, dithiothreitol (2 mM), MalNEt (2.5 mM) and *N,N'-o*-phenylenedimaleimide (67 μM) were added as indicated, in the presence of 1.5 μM nigericin ($[K^+]$ = 20 mM). The suspension was preilluminated in blue light for 30 s then $MgCl_2$ (to 5 mM) and ferredoxin (to 1 μM) were added together with cytochrome *c* (125 μM) where shown. Process 2 was estimated from the rate of light-driven O_2 evolution following addition of 250 μM $NADP^+$; processes 3 and 4 were measured from the rate of absorbance increase at 550 nm minus that at 540 nm upon illumination or addition of NADPH (250 μM), respectively. Absolute rates of the controls (in μequiv./mg chlorophyll per h) are shown in brackets; other values are expressed as percentages thereof. Similar data were obtained when preillumination in red light or preincubation with NADPH were used to establish inhibition. It was also immaterial whether ferredoxin was present during the pretreatment or was added later, as above.

Addition	Process		
	2 ($H_2O \rightarrow NADPH^+$)	3 ($H_2O \rightarrow$ cytochrome *c*)	4 (NADHP → cytochrome *c*)
None	100 (184)	100 (148)	100 (69)
MalNEt	0	99	23
MalNEt + dithiothreitol	97	98	79
Phenylenedimaleimide	31	91	31
Phenylenedimaleimide + dithiothreitol	103	82	110

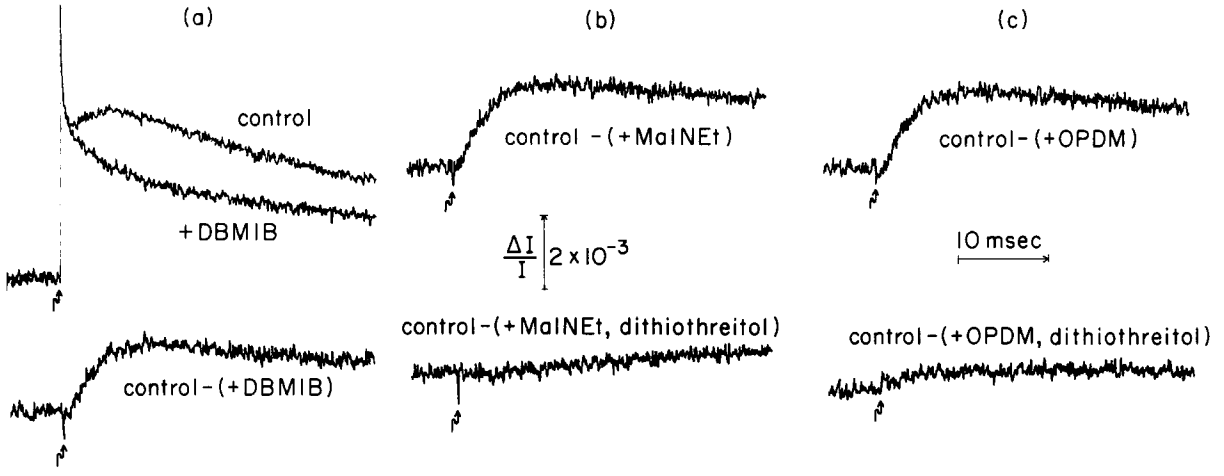

Fig. 4. Effect of maleimides on the flash-induced absorbance change at 518 nm. Chloroplasts (120 μg chlorophyll) were shocked in 1.5 ml of 1 mM Tricine, pH 8.1, containing 10 mM $MgCl_2$, 1 000 U catalase and 3.3 μM ferredoxin; 1.5 ml of double-strength reaction mixture (minus $MgCl_2$) and 1.3 μM nigericin were then added. After mixing, MalNEt (5 mM) or *N,N'-o*-phenylenedimaleimide (65 μM) with or without dithiothreitol at half the maleimide concentration, or DBMIB (0.7 μM) were added. The samples were given continuous red (Corning 2-58) illumination for 30 s followed by 15 saturating flashes (see Materials and Methods) before averaging the absorbance transients at 518 nm induced by 128 flashes at 0.5 Hz. The upper traces in a show the form of the original data before subtraction to given difference traces such as those shown in a–c. $P518_s$ could be seen in MalNEt-treated samples if dithionite was added and the flash frequency decreased to 0.25 Hz, showing that the maleimide did not affect the reactions giving rise to $P518_s$, but rather inhibited at another point in the cycle.

TABLE III

EFFECT OF SULFO-DSPD ON DIAPHORASE AND CYTOCHROME *c* REDUCTASE ACTIVITIES OF SOLUBLE FERREDOXIN-$NADP^+$ REDUCTASE

Activities were assayed spectrophotometrically in 1.0 ml of standard reaction medium minus catalase. Present in the diaphorase assay were 100 μM dichlorophenolindophenol, 100 μM NADPH, 1 μM ferredoxin where indicated and 200 pmol of purified reductase; dye reduction was monitored as the absorbance decrease at 600 nm minus that at 500 nm. Cytochrome *c* reduction was similarly monitored using the wavelength pair 550 nm minus 540 nm; assay media contained 10 μM cytochrome *c*, 100 μM NADPH, 1 μM ferredoxin and 40 pmol of reductase. Reactions were started following at 2.5 min incubation by adding NADPH or (values in parentheses) reductase. Sulfo-DSPD, 600 μM where shown. Initial rates given are nequiv./min.

	Process		
	NADPH → DCIP (−ferredoxin)	NADPH → DCIP (+ferredoxin)	NADPH → cytochrome *c* (+ferredoxin)
Control	260 (300)	350 (320)	1760 (790)
+sulfo-DSPD	20 (230)	20 (290)	30 (520)

N,N'-o-phenylenedimaleimide, whereas photoreduction of cytochrome *c* is not. Dithiothreitol protects against inhibition as seen in earlier studies [20,36].

Fig. 4 shows the effect of the maleimides on $P518_s$ under conditions comparable to those above. Fig. 4a illustrates the known inhibition of $P518_s$ by DBMIB [15] and the difference trace obtained by subtraction of inhibited and control traces; essentially identical difference traces were obtained when MalNEt (Fig. 4b) or *N,N'-o*-phenylenedimaleimide (Fig. 4c) was substituted in place of DBMIB. Dithiothreitol substantially protected against the maleimide inhibitors. These inhibitory actions of the maleimides of $P518_s$ support the findings with sulfo-DSPD and imply the participation of ferredoxin-$NADP^+$ reductase in cyclic electron transport.

Discussion

Adequate justification exists for use, under the conditions described here, of the electrochromic shift ($P518_s$) as an indicator of cyclic electron flow: $P518_s$ was mostly lost when intact chloroplasts

were osmotically ruptured in the absence of Mg^{2+}, but was retained upon breakage into buffered 5 mM $MgCl_2$ [3]; it could also be restored by addition of ferredoxin and Mg^{2+} (Fig. 1). Other indicators of cyclic electron flow such as cytochrome *b*-563 and *f* turnover, proton gradient formation and photophosphorylation were restored in conjunction with $P518_s$ (data not shown). Earlier results [3] suggested that the ferredoxin involved in cyclic flow is bound to the membrane by Mg^{2+}, as might be predicted from its strong anionic charge [39], since its loss in Mg^{2+}-deficient suspensions is primarily responsible for the decline in cyclic turnover.

The slow 518 nm response resembled cytochrome *c* photoreduction (process 3) in its dependence on ferredoxin concentration (Fig. 2), much more ferredoxin being required for comparable restoration of processes 2 and 4 [27]. Ferredoxin and $NADP^+$ form a 1 : 1 : 1 complex with solubilized ferredoxin-$NADP^+$ reductase [29–31] with binding constants of $1.6 \cdot 10^5$ and $9.5 \cdot 10^3$ M^{-1}, respectively [30]; interaction between these binding sites is not seen in the solubilized enzyme [30]. However, a conformational effect of $NADP^+$ or NADPH on the reductase might not be transmitted to the ferredoxin-binding site after solubilization of the enzyme, owing to consequent increase in molecular flexibility. Alternatively or additionally, pyridine nucleotides might influence the environment of the ferredoxin-binding site by inducing minor conformational changes in CF_1 (which effectively screens the reductase from antibodies [40,41]) or in the supporting membrane, with consequent decrease in the binding constant for ferredoxin. Work outside the scope of the present study is needed to test the above possibilities.

The data of Table I conflict with claims [42,43] that endogenous cyclic phosphorylation is sensitive to an antibody raised against chloroplast cytochrome *f* reductase (presumed identical with ferredoxin-$NADP^+$ reductase); the supporting results [42,43] show, however, less than 60% inhibition of phosphorylation at the highest antibody titer studied, and do not include comparable data for its effect on process 2, 3 or 4. Furthermore, it was found elsewhere [25] that whereas antibody prepared against ferredoxin inhibited reduction of cytochrome *b*-563 by PS I, antibody against the reductase did not. Indeed, the latter may actually accelerate photophosphorylation rates measured under cycling conditions [28]. The conclusion, however, that reductase is not involved in cyclic flow [25] overlooks the possibility that cyclic flow through the reductase may be unaffected by the status of the principal antigenic site. The results shown in Table I are consistent with a selective blocking of NADP binding by antibody, without associated effect on electron transport from the reductase to an acceptor involved in cyclic electron flow and reduction of cytochrome *b*-563 [25].

In contrast to the sensitivity pattern seen in Table I, $P518_s$ correlates with processes 2 and 4, but not with 3, in its sensitivity to sulfo-DSPD (Fig. 3). The failure of sulfo-DSPD to inhibit photoreduction of cytochrome *c* (Fig. 3) suggests that at least under the conditions used here, sulfo-DSPD does not inhibit between PS I and ferredoxin. Although it may interfere with electron transfer between ferredoxin and reductase by blocking their complexation [29–33], its inhibition of the diaphorase activity of purified reductase (Table III) suggests a principle interaction with the reductase only.

The substituted maleimides appear to inhibit catalytic functions of the reductase, since MalNEt and *N,N'-o*-phenylenedimaleimide inhibit processes involving $NADP^+$/NADPH but do not inhibit photoreduction of cytochrome *c* (Table II). The maleimides react with a sulfhydryl group on the reduced form of the solubilized reductase [43] and the resulting loss of catalytic activity possibly results from release of the FAD coenzyme [38].

The sensitivity of $P518_s$ to sulfo-DSPD and to maleimides (Fig. 4) shows that a surface component on the oxidizing side of ferredoxin, presumably the reductase, participates in cyclic flow. The notion that this species is not the reductase would require that sulfo-DSPD and maleimides, which have quite different inhibitory characteristics and probable modes of action, also block cyclic transfer from ferredoxin (or its reductant) toward an unidentified component within the membrane; this possibility cannot be eliminated by the data presented. Since, however, the reductase indisputably forms a complex with ferredoxin [29–33] and has the appropriate inhibitor sensitivity, it is the best candidate for the branch-point between cyclic and linear electron flow

at the reducing side of PS I.

Flash spectrophotometric studies of intact *Chlorella* [44,45] are claimed to show rapid reduction of ferredoxin-$NADP^+$ reductase and its reoxidation by the cyclic route [22]. The further conclusion is reached that reduction of the enzyme does not involve the ferredoxin iron-sulfur centers [45]. It is probably unsafe to assume, however, that electrons must leave the reductase via the cyclic route when PS II is inhibited, since cyclic turnover requires correct redox poise [17]. Moreover, at low flash frequencies in intact cells, electrons slowly entering intersystem pools [20] could possibly support linear flow, with endogenous $NADP^+$ or O_2 serving as the terminal oxidant. A further difficulty in relating these studies to the present work could arise from absence of evidence showing that *Chlorella* cells in which ferredoxin iron-sulfur centers appeared not to function, in fact contained ferredoxin. *Chlorella* normally contains flavodoxin equivalent to about 10% of the ferredoxin content [46]; in iron-deficient cultures ferredoxin is totally supplanted by flavodoxin [47]. Under such conditions, flash spectrophotometry would reveal not only an absence of absorbance changes due to ferredoxin, but the kinetics of flavodoxin turnover superimposed on those of ferredoxin-$NADP^+$ reductase.

Consideration of ferredoxin-$NADP^+$ reductase as a branch-point at which an electron is directed either away from, or back into the thylakoid membrane, underscores our need for more information on its catalytic site and conformation. Kinetic data for turnover of the reductase, following flash excitation of PS I in isolated chloroplasts, should provide a means of confirming the dual function proposed here, and should permit study of the competitive interaction between the cyclic and linear pathways.

Acknowledgements

Catherine Chia provided skilled technical assistance. Sulfo-DSPD was a kind gift from Dr. A. Trebst, Ruhr University, Bochum, F.R.G. Thanks are also due to Dr. R.L. Hamill of Eli Lilly Laboratories, for a supply of nigericin. This research was carried out at Brookhaven National Laboratory under the auspices of the U.S. Department of Energy.

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