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CYTOCHROME *b*-563 REDOX CHANGES IN INTACT CO_2-FIXING SPINACH CHLOROPLASTS AND IN DEVELOPING PEA CHLOROPLASTS

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SUMMARY

Intact spinach chloroplasts, capable of high rates of photochemical oxygen evolution with CO_2 as electron acceptor (120–350 μmol O_2 mg chlorophyll$^{-1} \cdot h^{-1}$) were examined for cytochrome redox changes. The response of the cytochromes in intact chloroplasts to oxidants and reductants appears to be governed by the permeability of the chloroplast envelope. The low potential cytochromes (*b*-559_{LP} and *b*-563) were more slowly reduced at 25 °C by dithionite than is the case with broken chloroplasts. At 0 °C, the reduction of the low potential cytochromes in intact chloroplasts was extremely slow. The chloroplast envelope is impermeable to ferricyanide, slowly permeable to ascorbate and rapidly permeable to reduced dichlorophenolindophenol.

Light-induced redox changes of cytochrome *b*-563 in intact chloroplasts were examined both at 0° and 25 °C. A red/far-red antagonism on the redox changes of cytochrome *b*-563 was observed at 0° C under anaerobic conditions. 3-(3,4-dichlorophenyl)-1, 1-dimethylurea (DCMU) inhibited the photoreduction of cytochrome *b*-563 in red light following far-red illumination. The photooxidation of cytochrome *b*-563 under anaerobic conditions was not influenced by DCMU or 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB). The photoreduction of cytochrome *b*-563 under aerobic conditions was much less efficient than its photooxidation under anaerobic conditions.

Developing pea chloroplasts showed much greater light-induced redox changes of cytochrome *b*-563 than did intact spinach chloroplasts.

Our data are consistent with the view that cytochrome *b*-563 functions on a cyclic pathway around Photosystem I, but it appears that cyclic flow is sensitive to the relative poising of the redox levels of cytochrome *b*-563 and the components of the non-cyclic pathway.

Abbreviations: CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; Cl_2Ind, 2-6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl *p*-phenylenediamine.

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INTRODUCTION

Chloroplasts contain three types of cytochrome: cytochrome *f*, (*c*-type α-band at 554 nm), cytochrome *b*-563 and cytochrome *b*-559 [1, 2]. It is generally considered that cytochrome *f* functions as an electron carrier between the two photosystems and close to Photosystem I, but there is no agreement about the roles of the *b* cytochromes in chloroplasts. The situation is complicated by the existence of two redox forms of cytochrome *b*-559 in chloroplasts [2, 3]: cytochrome $b\text{-}559_{HP}$ with a redox potential of +0.37 V at pH 6.5 and 7.5 and cytochrome $b\text{-}559_{LP}$ with a redox potential of +0.065 V [4]. From fractionation studies of chloroplasts disrupted with detergents, it was concluded that cytochrome $b\text{-}559_{HP}$ is localized in Photosystem II [1], whereas cytochrome $b\text{-}559_{LP}$ is closely associated with cytochromes *f* and *b*-563 in Photosystem I [5]. To complicate matters further, cytochrome $b\text{-}559_{HP}$ in chloroplasts is converted to a form of lower potential by aging, mild heating or incubation with the detergent, Triton X-100 [6].

Arnon et al. [7] suggested that cytochrome *b*-563 functions in cyclic phosphorylation associated with Photosystem I, because of the sensitivity of ferredoxin-mediated cyclic phosphorylation to antimycin A. Studies with mutants of *Chlamydomonas reinhardi* [8] also suggested a role for cytochrome *b*-563 in cyclic phosphorylation. There are some data, however, which indicate that cytochrome *b*-563 sometimes functions on a pathway between the photosystems. Ikegami et al. [9] observed that cytochrome *b*-563 was photooxidized by Photosystem I and photoreduced by Photosystem II in *Euglena* chloroplasts at a nonphysiological pH of 9.5 while Hind and Olson [10] suggested that cytochrome *b*-563 is a component of a non-phosphorylating pathway between the photosystems. More recent work [11–15] is consistent with the view that cytochrome *b*-563 functions on a cyclic pathway around Photosystem I, but it does not exclude the possibility that cytochrome *b*-563 also functions on a pathway connected to Photosystem II.

It seems possible that the discrepancies in the light-induced redox behaviour of the *b*-cytochromes in isolated chloroplasts are due to differences in preparative and assay conditions. We have therefore examined light-induced absorbance changes in intact spinach chloroplasts, with high rates of CO_2 fixation. Some comparative experiments on the redox changes of cytochrome *b*-563 in developing pea plastids are also included.

MATERIALS AND METHODS

Spinach plants were grown in water culture in a glasshouse [16]. Pea seedlings were grown in vermiculite in darkness at 25 °C for 10 days, and then illuminated with white light of 850 foot-candles (12 600 erg · cm^{-2} · s^{-1} as measured by a thermopile) for 3 h [17]. Intact spinach chloroplasts, capable of high rates of CO_2 fixation, were isolated as described previously [18]. Developing plastids were obtained from greening pea leaves by the same method. Reactions were usually performed in a medium containing 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.8 mM KH_2PO_4, 10 mM NaCl, 50 mM HEPES buffer, pH 7.6 and 300 mM sorbitol. Oxygen evolution was measured polarographically at 20 °C with a Clark-type electrode from Rank

Bros., Cambridge. Actinic light was provided by a 250 W lamp (Philips Photoflood) and filtered through a 5 cm layer of water and a sheet of red perspex.

Oxidized minus reduced difference spectra were recorded on a Cary Model 14R spectrophotometer equipped with a scattered-transmission accessory [19]. The chlorophyll concentration for the cytochrome measurements was 60–75 μg/ml and the optical pathlength was 1 cm. Cytochromes *f* and *b*-559$_{HP}$ were determined from ferricyanide oxidized minus hydroquinone reduced difference spectra with osmotically shocked chloroplasts, since ferricyanide does not penetrate intact chloroplasts. Cytochromes *b*-563 and *b*-559$_{LP}$ were measured with both intact and broken chloroplasts from dithionite reduced minus hydroquinone reduced difference spectra. Ferricyanide and hydroquinone were added to a final concentration of 2 mM and dithionite to a concentration of 3–10 mM. For cytochrome determinations on developing pea plastids, the plastids were purified on a Ludox gradient, and osmotically shocked for 5 s.

Light-induced absorbance changes were measured either with a Cary spectrophotometer, or with an Aminco-Chance dual wavelength spectrophotometer equipped with a side illumination system which allowed the use of one or two actinic light beams. Red light was provided by a tungsten iodine lamp, and a 655 nm narrow band interference filter (Balzer), or a combination of a Corning 2-60 glass filter and a K5 or K6 broad band interference filter (Balzer). Corning filters 2-60 or 2-64 were used to obtain a broad band of red plus far-red light. Far-red light was obtained with an RG 715 cut off filter (Schott/Mainz) or a combination of an RG 695 cut off filter and a 714 nm narrow band interference filter (Balzer). In all cases, a 32 mm layer of water was used as a heat filter. Light intensities were measured with a silicon photocell, which was calibrated against a thermopile (Hewlett-Packard). In the determination of quantum requirements, the absorbance of samples was measured in a large Ulbricht sphere.

Anaerobic conditions were produced either with sodium dithionite or glucose (5 mM) plus glucose oxidase (approx. 20 μg in a final volume of 3.3 ml). Catalase (10–100 μg/3.3 ml) was added in some experiments to prevent peroxide formation.

The following 3-wavelength equations were used for the determination of cytochromes (μmol · ml^{-1}) from the observed difference spectra.

$$\text{cyt } b\text{-}559 = 0.0968\, E_{559} - 0.0299\, E_{554} - 0.0541\, E_{563}$$
$$\text{cyt } f \quad = 0.0577\, E_{554} + 0.0225\, E_{563} - 0.0402\, E_{559}$$
$$\text{cyt } b\text{-}563 = 0.083\, E_{563} + 0.0182\, E_{554} - 0.059\, E_{559}$$

These equations are based on the following milli-molar extinction coefficients: cytochrome *f*, $\varepsilon^{1\,cm}_{554\,nm} = 22$, $\varepsilon^{1\,cm}_{559\,nm} = 6.8$; cytochromes *b*-559$_{HP}$ and *b*-559$_{LP}$, $\varepsilon^{1\,cm}_{559} = 20$, $\varepsilon^{1\,cm}_{563} = 12.2$, $\varepsilon^{1\,cm}_{554} = 9.1$; cytochrome *b*-563, $\varepsilon^{1\,cm}_{563} = 20$, $\varepsilon^{1\,cm}_{559} = 11.2$.

Application of these equations neglects interference by other compounds which absorb in the same region as the cytochromes and respond to changes in redox conditions *C*-550 is reduced by dithionite and also by light absorbed by Photosystem II [20]. Whereas reduction of the low potential cytochromes results in an increase in absorption, reduction of *C*-550 decreases the absorption. The relative decrease in absorption due to reduction of *C*-550 at 554, 559 and 563 nm is 1.0:0.8:0.55. The cytochrome estimations using the 3-wavelength equations, therefore, are only semiquantitative; the values obtained represent lower limits in the case of reduction

of cytochromes *b*-563 and *b*-559$_{LP}$ by dithionite or by red light, and upper limits for photooxidation of cytochrome *f*.

Light-induced changes in fluorescence yields were measured as described previously [21]. Chlorophyll was determined on acetone extracts of chloroplasts [22].

RESULTS

Activity of intact chloroplasts

A high percentage (75–95 %) of the spinach chloroplasts in fresh preparations were intact and retained their envelopes. Photochemical 0_2 evolution with bicarbonate (CO_2) as electron acceptor was assayed routinely; values ranged from 120 to 350 μmol O_2 · chlorophyll^{-1} · h^{-1}. The chloroplasts reduced added 3-phosphoglycerate at high rates (160–450 μmol O_2 · mg chlorophyll^{-1} · h^{-1}) and oxaloacetate reduction with coupled chloroplasts gave rates ranging from 40 to 160 μmol · mg chlorophyll^{-1} · h^{-1}. Oxaloacetate reduction was stimulated by uncouplers, while phosphoglycerate and CO_2 reductions were completely inhibited. Fig. 1 shows oxygen electrode traces with intact chloroplasts: with CO_2 reduction, there was a characteristic lag phase [23] which was absent with phosphoglycerate or oxaloacetate reductions. The chloroplasts were surprisingly stable; after 24 h at 0 °C, rates of CO_2 reduction sometimes were 50 % of the rate with the freshly-prepared chloroplasts.

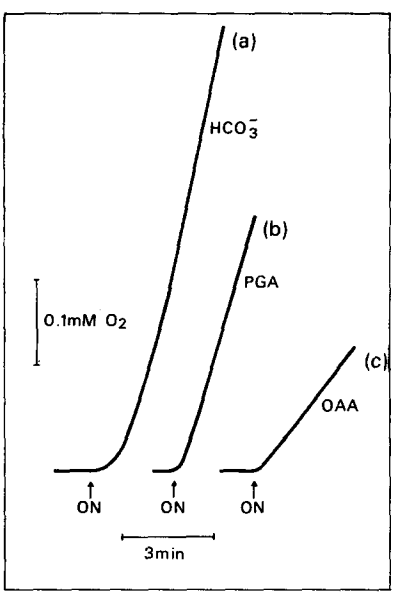

Fig. 1. Oxygen evolution by spinach chloroplasts during reduction of (a) bicarbonate (CO_2) (4 mM), (b) 3-phosphoglycerate (2 mM) and (c) oxaloacetate (1 mM), as measured polarographically at 20 °C with an oxygen electrode. Chlorophyll concentration, 38 μg/ml; pH, 7.6. Rates of reduction were: CO_2, 280 μmol · mg chlorophyll^{-1} · h^{-1}; 3-phosphoglycerate, 418 μmol · mg chlorophyll^{-1} · h^{-1}; oxaloacetate, 155 μmol · mg chlorophyll^{-1} · h^{-1}.

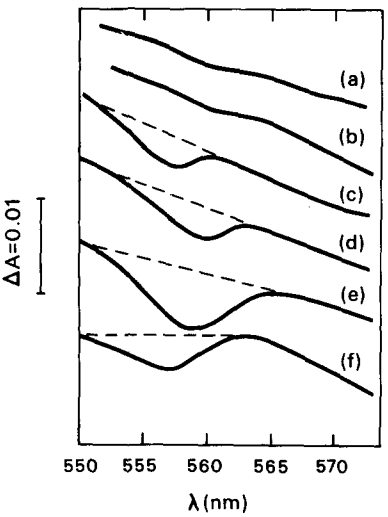

Fig. 2. Effect of ferricyanide and light on the redox state of the "high" potential cytochromes in intact spinach chloroplasts and detergent-treated chloroplasts. (a) Chloroplasts suspended in isotonic medium, untreated and in the dark. (b) 1 mM ferricyanide, dark. (c) 1 mM ferricyanide, far-red light. (d) 1 mM ferricyanide +2 μM FCCP, red light. (e) 1 mM ferricyanide +2 μM FCCP+0.05 % Triton X-100, dark. (f) 1 mM ferricyanide+2 μM FCCP +0.05 % Triton X-100, after 30 min in dark. Chlorophyll concentration, 75 μg/ml.

Accessibility of cytochromes to oxidants and reductants in intact chloroplasts

Cytochrome *f* and cytochrome *b*-559$_{HP}$ were in the reduced state in freshly prepared intact chloroplasts as shown by the lack of further reduction of these cytochromes by hydroquinone. Penetration of hydroquinone across the chloroplast envelope was indicated by the stimulation of dark reduction of photooxidized cytochrome *f* and, in the presence of 2 μM FCCP, of ferric cytochrome *b*-559$_{HP}$.

Ferricyanide did not oxidize cytochrome *f* and cytochrome *b*-559$_{HP}$ in intact chloroplasts (Fig. 2, curve b) due presumably to the impermeability of the chloroplast envelope to ferricyanide. Light, however, caused cytochrome *f* oxidation (Fig. 2, curve c). This contrasts with the behaviour of osmotically shocked chloroplasts where ferricyanide oxidized cytochrome *f* and light did not cause any further oxidation. In the presence of 2 μM FCCP, cytochrome *b*-559$_{HP}$ in intact chloroplasts was photooxidized (curve d) by red light. Only after the chloroplast envelope was destroyed by detergent or by osmotic shock did ferricyanide oxidize the high potential cytochromes (curve e). The band of ferrous cytochrome *b*-559 slowly disappeared on prolonged treatment with detergent (curve f) because of the autoxidation of cytochrome *b*-559$_{HP}$ in the reference curvette. A comparison of curves c and d with curve e indicates that light did not completely photooxidize cytochrome *f* or cytochrome *b*-559$_{HP}$.

In contrast to osmotically shocked chloroplasts, intact chloroplasts did not photoreduce ferricyanide and they exhibited variable fluorescence and a high steady-state level of fluorescence in the presence of ferricyanide. Thus, again it is apparent that ferricyanide did not pentrate intact chloroplasts. In a modification of the method described previously [24] this lack of penetration of ferricyanide was used to estimate the

fraction of broken chloroplasts in chloroplast preparations. The extents of oxidation of cytochromes *f* and *b*-559$_{HP}$ by ferricyanide were measured before and after treatment of a chloroplast preparation with 0.05 % Triton X-100 or brief sonication. Chloroplasts were made anaerobic by glucose plus glucose oxidase before addition of Triton X-100 to prevent autoxidation of cytochrome *b*-559$_{HP}$ in the reference cuvette. (Cytochrome *b*-559$_{HP}$ is converted to an autoxidizable form of lower potential by treatment of chloroplasts with Triton X-100.)

The low potential cytochromes (*b*-563 and *b*-559$_{LP}$) which are in the oxidized state in untreated chloroplasts, were reduced by addition of sodium dithionite to intact chloroplasts at 25 °C; their rate of reduction, however, was slower in intact chloroplasts than in shocked chloroplasts. With intact chloroplasts, reduction of cytochrome *b*-563 by dithionine often was not complete even after 20 min, and further reduction was usually observed on addition of 0.05 % Triton X-100. Cytochrome *b*-559$_{LP}$ was reduced more rapidly by dithionite than was cytochrome *b*-563, in agreement with the work of Bendall [2, 3]. At 0 °C, the reduction of the low potential cytochromes in intact chloroplasts by dithionite was very slow (Fig. 3).

The slow reduction by dithionite of the low potential components of the electron transport chain of intact chloroplasts was also indicated by fluorescence measurements. At 0 °C, fluorescence of intact chloroplasts was quenched by low intensity far-red (120 erg · cm^{-2} · s^{-1}) even in the presence of 5 mM sodium dithionite (Fig. 4). Illumination with red light (650 mm) in the presence of 0.27 mM O_2 caused a large biphasic increase in the fluorescence yield, which, however, was quenched considerably by the addition of 3-phosphoglycerate or oxaloacetate as electron acceptor. In contrast with its effect on intact chloroplasts dithionite produced a high fluorescence yield in broken chloroplasts in far-red light at 0 °C by reducing the electron carriers on the acceptor side of Photosystem II [25] (Fig. 5). With intact chloroplasts at 25 °C, oxidation of the electron transport chain by far-red light is too slow to compete with the faster reduction by dithionite. Consequently, a high fluorescence yield is obtained immediately on turning on the red light.

Cytochromes in intact chloroplasts interacted only to a limited extend with either benzoquinone or dithiothreitol. Benzoquinone caused a small degree of oxidation of cytochrome *f* and cytochrome *b*-559$_{HP}$ at pH 7.6 and somewhat more

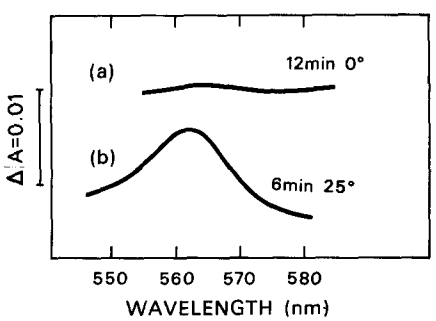

Fig. 3. Reduction of low potential cytochromes in intact spinach chloroplasts by 5 mM dithionite. (a) Dithionite reduced minus hydroquinone reduced difference spectrum after 12 min at 0 °C. (b). Difference spectrum of the same sample after warming to room temperature for 6 min. Chlorophyll concentration, 75 μg/ml.

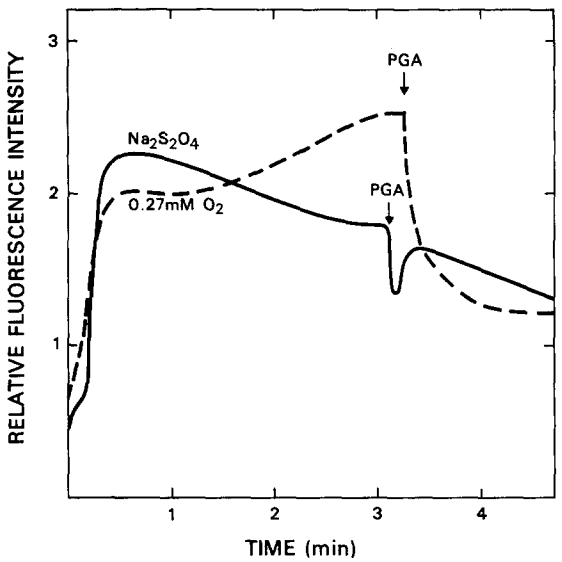

Fig. 4. Fluorescence kinetics of intact spinach chloroplasts at 0 °C. (- - - -) in the presence of 0.27 mM oxygen; (—) in the presence of 5 mM dithionite. The chloroplasts were preilluminated for 5 min with 715 nm light (120 erg · cm^{-2} · s^{-1}) before excitation with 650 nm light (360 erg · cm^{-2} · s^{-1}). 2 mM 3-phosphoglycerate (PGA) was added where indicated. Emission wavelength, 685 nm.

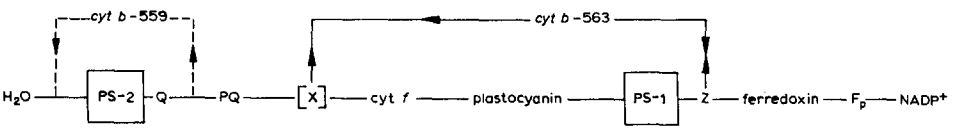

Fig. 5. Scheme for photosynthetic electron transport, showing cytochrome b_{563} on a cyclic pathway around Photosystem I. The direction of flow around the cycle is dependent on the redox states of cytochrome *b*-563 and the components Z and X, where the noncyclic and cyclic pathways diverge or converge. Q is the primary acceptor for Photosystem II; in its oxidized state Q quenches fluorescence. PQ is plastoquinone. Z is the primary acceptor for Photosystem I and may be identical to a bound ferredoxin. F_P is a flavoprotein (ferredoxin-NADP reductase). [X] is a hypothetical carrier where the cyclic and noncyclic pathways converge. Far-red light is adsorbed predominantly by Photosystem I, while red light is adsorbed by both photosystems.

TABLE I

CYTOCHROME CONTENTS OF INTACT SPINACH CHLOROPLASTS

Experiment number	mol/1000 mol chlorophyll			
	f	*b*-559$_{HP}$	*b*-559$_{LP}$	*b*-563
1	1.7	3.2	2.7	4.8
2	2.5	3.3	2.8	3.7
3	2.7	2.3	2.7	3.8
4	1.5	3.7	2.3	5.3
5	1.8	3.0	1.2	7.0
6	1.5	3.9	2.6	4.9
Average	2.0	3.2	2.4	4.9

at pH 6.1. Dithiothreitol ($E'_0 = -0.32$ V), which completely reduces soluble mammalian cytochrome *c*, caused only a partial reduction of cytochrome b-559_{LP} and it had no effect on the redox state of cytochrome *b*-563. Ascorbate did not significantly reduce cytochrome b-559_{LP}. The dark reduction of photooxidized cytochrome *f* and photooxidized cytochrome b-559_{HP} (in the presence of FCCP) by ascorbate was very slow, although the reduction of these cytochromes by reduced dichlorophenolindophenol was rapid.

Table I shows cytochrome contents of intact chloroplasts. Data on cytochromes *f* and b-559_{HP} were obtained after shocking the chloroplasts osmotically. Errors attributable to the sieve effect were small as indicated by the inability of Triton X-100 to alter, in the absence of oxygen, the shape and height of the redox difference spectra. However, there remains some uncertainty in the case of the low potential cytochromes due to the interference caused by the reduction of C-550 by dithionite (see Materials and Methods).

Photooxidation of cytochrome f

In the presence of 3-phosphoglycerate and O_2, 60 % of the cytochrome *f* of intact chloroplasts was oxidized by strong far-red light at 27 °C (Table II). There was less oxidation (40 %) by 3-phosphoglycerate when oxygen was removed by glucose plus glucose oxidase. Omission of both 3-phosphoglycerate and O_2 further decreased oxidation (20 %). In the presence of dithionite, photooxidation of cytochrome *f* was not observed even in the presence of 3-phosphoglycerate, but lowering the temperature to 0 °C decreased the effectiveness of dithionite in intact chloroplasts and permitted substantial photooxidation of cytochrome *f* in the presence of 3-phosphoglycerate or oxaloacetate. In general, a decrease in temperature enhanced cytochrome *f* oxidation, thus indicating a temperature-sensitive rate-limiting step between Photosystem II and cytochrome *f* (Fig. 5).

Light-induced redox changes of cytochrome b-563

At 27 °C, there was a slight reduction of cytochrome *b*-563 (approx. 5 %) when intact chloroplasts were exposed to red or far-red light (Fig. 6a) in the presence of oxygen and 3-phosphoglycerate. The extent of the photoreduction of cytochrome *b*-563 by red light in the presence of 3-phosphoglycerate was increased significantly by the

TABLE II

EXTENT OF PHOTOOXIDATION OF CYTOCHROME *f* IN INTACT SPINACH CHLOROPLASTS

The intensity of the far-red actinic light was 32 kerg · cm^{-2} · s^{-1}.

Electron acceptors presence	Cytochrome *f* oxidized (mol/1000 mol chlorophyll)			
	Phosphoglycerate + O_2	Phosphoglycerate[a]	nil[a]	Phosphoglycerate[b]
Temp. 27 °C	1.2	0.8	0.4	0
Temp. 0 °C	1.5	–	–	0.8

[a] Oxygen removed with glucose plus glucose oxidase.
[b] Oxygen removed with dithionite.

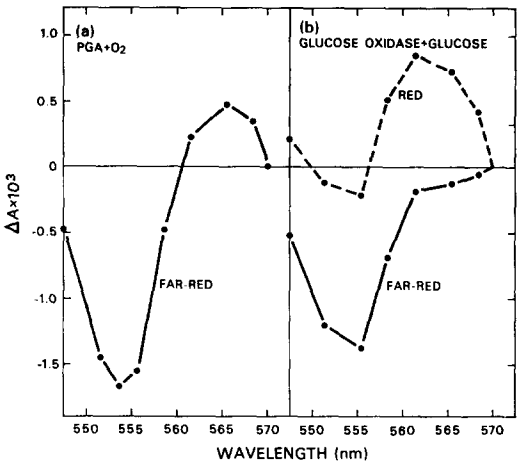

Fig. 6. Light minus dark difference spectra of intact spinach chloroplasts at 27 °C. Reference wavelength, 570 nm. (a) In the presence of 2 mM 3-phosphoglycerate and 0.27 mM O_2. (b) In the presence of glucose plus glucose oxidase. Actinic light intensities: (—), far-red (Schott, R G 715), 30 kerg · cm^{-2} · s^{-1}; (- - - -) red, 32 kerg · cm^{-2} · s^{-1}. Chlorophyll concentration, 60 μg/ml.

removal of oxygen with glucose plus glucose oxidase (Fig. 6b): under these conditions far-red light was ineffective in reducing cytochrome *b*-563, and it even caused some oxidation.

Light-induced redox changes of cytochrome *b*-563 in intact chloroplasts were also studied at 0 °C. A small percentage of cytochrome *b*-563 was reduced in the dark on making the chloroplasts anaerobic with glucose plus glucose oxidase or dithionite. In the absence of an electron acceptor, such as 3-phosphoglycerate, illumination of the anaerobic chloroplasts either with monochromatic red light (655 nm) or a broad band of red light caused a slight photooxidation of cytochrome

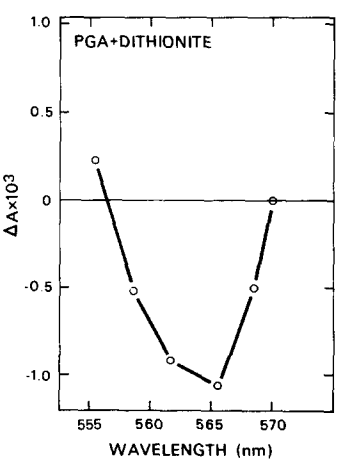

Fig. 7. Light minus dark difference spectrum of intact spinach chloroplasts at 27 °C in the presence of 2 mM 3-phosphoglycerate and approx. 5 mM dithionite. Reference wavelength, 570 nm. Chlorophyll concentration, 60 μg/ml. High intensity red light, 260 kerg · cm^{-2} · s^{-1} was used.

b-563. Oxidation was also induced by far-red light (715 nm). The very small extent of oxidation was considerably enhanced if the low potential cytochromes were reduced by dithionite at room temperature. A light minus dark difference spectrum is shown in Fig. 7. Far-red light was usually slightly more effective than red light in photooxidizing cytochrome *b*-563. The extent of the photooxidation at 25 °C, when most of the cytochrome *b*-563 was reduced by dithionite, was no greater than 15 % even with high intensity actinic light, despite the fact that the dark reduction of cytochrome *b*-563 in intact chloroplasts is slow and unable to compete with the rapid photooxidation.

Effect of inhibitors

The effect of DCMU on cytochrome *b*-563 reduction in intact chloroplasts was dependent on experimental conditions. Reduction by strong red light at 0 °C in the presence of 3-phosphoglycerate or in aged chloroplasts was largely or completely inhibited by 10 μM DCMU. In freshly isolated chloroplasts reduction of cytochrome *b*-563 by red light at 25 °C in the presence of oxygen was scarcely affected by DCMU and reduction by far-red light sometimes was stimulated. It might appear that under certain conditions electrons for cytochrome *b*-563 reduction are provided by Photosystem II, but there is an alternative explanation (see Discussion). DBMIB (30 μM) completely inhibited reduction of cytochrome *b*-563.

Photooxidation of cytochrome *b*-563 in the presence of dithionite was not influenced by DBMIB or DCMU.

Red/far-red antagonism on redox state of cytochrome b-563

Intact chloroplasts were made anaerobic with dithionite at 0 °C in the presence

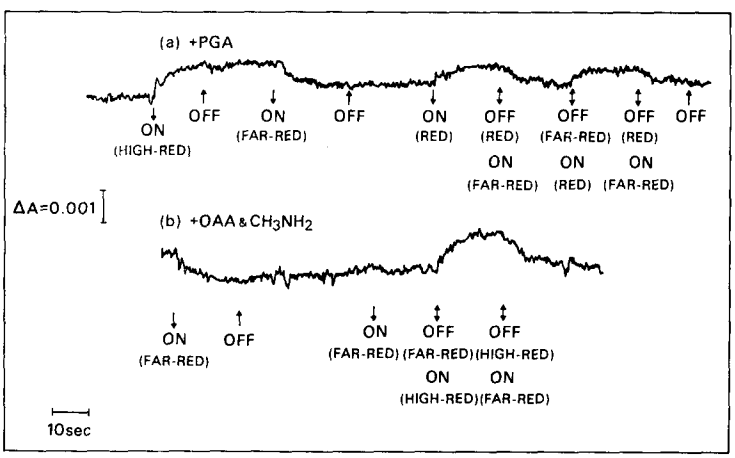

Fig. 8. Red/far-red antagonism by intact spinach chloroplasts at 0 °C in the presence of approx. 5 mM dithionite and either (a) 2 mM 3-phosphoglycerate or (b) 2 mM oxaloacetate and 10 mM methylamine. (a) The chloroplasts were preilluminated with far-red light followed by a dark period. (b) The chloroplasts were preilluminated with red light followed by a dark period. Light intensities; high intensity red (Corning 2-60), 260 kerg · cm^{-2} · s^{-1}; red (Balzer K6), 65 kerg · cm^{-2} · s^{-1}; far-red (Schott RG 715) 35 kerg · cm^{-2} · s^{-1}. Sample wavelength, 563 nm; reference wavelength, 570 nm.

of 3-phosphoglycerate (Fig. 8a) or oxaloacetate (Fig. 8b). Strong red light caused some reduction of cytochrome *b*-563 (Fig. 8a). Its reoxidation on turning off the light was very slow indeed. A second illumination with red light caused a negligible change in absorbance (not shown). It is apparent that in the absence of oxygen, photoreduced cytochrome *b*-563 stayed largely reduced in the dark. Far-red light which in the presence of oxygen causes cytochrome *b*-563 reduction now reoxidized the cytochrome. Following the far-red illumination, there was little change in absorbance in the dark and a second illumination with far-red had little effect. Addition of DCMU inhibited these antagonistic cytochrome *b*-563 absorbance changes, by preventing the photoreduction in red light following far-red illumination. In the experiment shown in Fig. 8b, red illumination followed by a dark period preceded illumination with far/red.

In order to observe the red/far-red antagonism of cytochrome *b*-563 redox changes in intact chloroplasts, it was necessary to have anaerobic conditions and to add an electron acceptor such as 3-phosphoglycerate or oxaloacetate. The effect was seen best at 0 °C in the presence of dithionite; these conditions caused only a small degree of reduction of the low potential cytochromes (Fig. 3). Occasionally, it was seen in the presence of glucose plus glucose oxidase. The poor reproducibility under these conditions is probably due to the lower efficiency of the enzymic oxygen-trapping system.

The red/far-red antagonism of the redox behaviour of cytochrome *b*-563 was distinguished from that of cytochrome *f* by the intensity requirements, the kinetics of the changes and the difference spectra (Fig. 9). Red/far-red antagonism of the

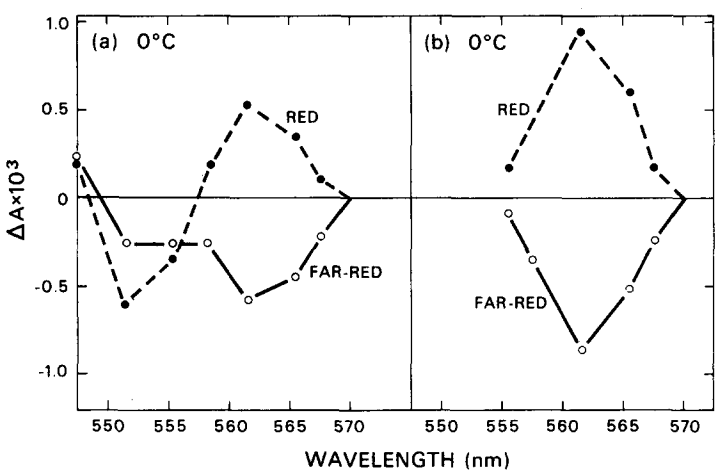

Fig. 9. Light minus dark difference spectra of red/far-red antagonistic absorbance changes in intact spinach chloroplasts at 0 °C in the presence of 2 mM 3-phosphoglycerate and dithionite. Reference wavelength, 570 nm. (a) (- - -) spectrum from illumination with high intensity red (Corning 2-60, 250 kerg · cm^{-2} · s^{-1}) after previous illumination with far-red light (Schott RG 695, 32 kerg · cm^{-2} · s^{-1}) and 15 s dark period. (—), spectrum from illumination with far-red (Schott RG 715, 32 kerg · cm^{-2} · s^{-1}) after previous illumination with high intensity red light and a 15 s dark period. (b) (- - -), spectrum from illumination with high intensity red (Corning 2-60) after previous illumination with far-red light (Schott RG 715). (—), spectrum from illumination with far-red (Schott RG 715) after previous illumination with high intensity red light. There were no intermittent dark periods as in (a).

TABLE III

QUANTUM REQUIREMENTS FOR THE PHOTOOXIDATIONS OF CYTOCHROMES IN INTACT SPINACH CHLOROPLASTS

The temperature was 25–29 °C.

Cytochrome	Additions	Wavelength actinic light (nm)	Quantum requirement		
			average	standard deviation	No. of expts.
b-563	approx. 5 mM dithionite and 2 mM phosphoglycerate	714	3.6	0.6	6
		655	6.3	2.2	6
f	20 μM DCMU, 0.27 mM O_2 2 mM phosphoglycerate or 2 mM oxaloacetate	714	4.0	0.9	11
		655	9.7	4.2	12
f	0.27 mM O_2, 2 mM phosphoglycerate	714	10	2.6	6
		655	∞	–	–

redox state of cytochrome *f* was not seen under these conditions, since oxidation o cytochrome *f* occurred in both strong red and far-red light.

Quantum requirements for cytochrome redox changes

Quantum requirements for the photooxidation of cytochrome *b*-563 an cytochrome *f* in intact chloroplasts are shown in Table III. The quantum requiremen for oxidation of cytochrome *b*-563, in the presence of dithionite and 2 mM 3-phos phoglycerate at 25 °C, was 3–4 in 714 nm light and 5–8 in 655 nm light. Cytochrom *f* photooxidation was measured using the same chloroplast preparation but in th absence of dithionite and the presence of DCMU; it showed quantum requirement of 3–5 in 714 nm light and 7–10 in 655 nm light. In the absence of DCMU, 10 quant per mol were needed at 714 nm. The quantum requirement for cytochrome *b*-56 reduction in the presence of oxygen was very high and it could not be determine with any degree of accuracy.

In the determination of these quantum requirements, corrections were mad for dark reductions of the cytochromes. However, the values obtained should no be considered as minimum requirements, since cyclic oxidoreductions would b expected to increase the quantum requirements.

Cytochrome redox changes in developing pea plastids

For comparison with intact spinach chloroplasts, we include some measurements on cytochrome *b*-563 redox changes in developing pea plastids. The developing pea plastids have the advantage of giving much greater light-induced absorbance changes. Plastids were isolated from pea seedlings which had been grown in the dark for 10 days and then illuminated for 3 h with white light. A chlorophyll *a*/*b* ratio o 8, determined as described previously [26], was found for both leaves and plastids. In contrast with 3 h plastids isolated in a sucrose phosphate medium [17] the plastids showed reasonable rates of oxygen evolution with ferricyanide or dichlorophenolindophenol as electron acceptors (Table IV). The developing pea plastids also showed

TABLE IV

PHOTOCHEMICAL ACTIVITIES OF DEVELOPING PEA PLASTIDS

Dark-grown pea seedlings were greened for 3 h and the plastids isolated as described in Materials and Methods.

Reaction	Oxygen (μmol · mg chlorophyll^{-1} · h^{-1})	μequivalent · mg chlorophyll^{-1} · h^{-1}
$H_2O \rightarrow Cl_2Ind$	145, 103, 99	290
$H_2O \rightarrow K_3Fe(CN)_6$	133	532
Ascorbate/TMPD → methyl viologen	699, 920, 828	1630
$H_2O \rightarrow$ 3-phosphoglycerate	35, 91, 38	
$H_2O \rightarrow$ oxaloacetate	60, 109	
$H_2O \rightarrow CO_2$	6.3	

TABLE V

CYTOCHROME CONTENT OF DEVELOPING PEA PLASTIDS

Seedlings were greened for 3 h. Plastids were isolated as described in Materials and Methods and purified on a Ludox gradient. Values: mol/1000 mol chlorophyll.

f	*b*-559$_{HP}$	*b*-559$_{LP}$	*b*-563
5.0	1.7	11.5	14.5

some oxygen evolution with 3-phosphoglycerate or oxaloacetate as substrates but not with bicarbonate (CO_2). The plastids exhibited good Photosystem I activity (photoreduction of methyl viologen with reduced *N,N,N',N'*-tetramethyl *p*-phenylenediamine as donor). Since oxygen evolution was observed with ferricyanide and with 3-phosphoglycerate and oxaloacetate, it is concluded that the pea plastid preparations contained both intact and broken plastids.

Cytochrome determinations of 3 h pea plastids (Table V) indicated a low proportion of cytochrome *b*-559$_{HP}$ relative to cytochromes *f*, *b*-559$_{LP}$ and *b*-563, This agrees with previous findings [4, 27] that cytochrome *b*-559$_{HP}$ is absent from etioplasts and is formed relatively slowly during chloroplast development.

A substantial photoreduction of cytochrome *b*-563 (up to 40 %) was observed with 3 h plastids at 27 °C in the presence of oxygen (Fig. 10a), but very high actinic light was needed to obtain a high extent of reduction. Far-red light, as well as red light, was effective in catalyzing the reduction of cytochrome *b*-563. The presence of diquat, an electron acceptor for Photosystem I, decreased the level of reduction. Photoreduction of cytochrome *b*-563 in red light was inhibited by DCMU and it could be substantially restored by ascorbate and Cl_2Ind (Table VI). DBMIB inhibited cytochrome *b*-563 reduction, but it was not as effective as DCMU.

In the presence of dithionite, there was photooxidation of cytochrome *b*-563 (Fig. 10b). Lowering the temperature to 0 °C increased the extent of the photooxidation (Fig. 11). Neither DCMU or DBMIB influenced the photooxidation of cytochrome *b*-563 in the presence of dithionite. A photooxidation of cytochrome *f*

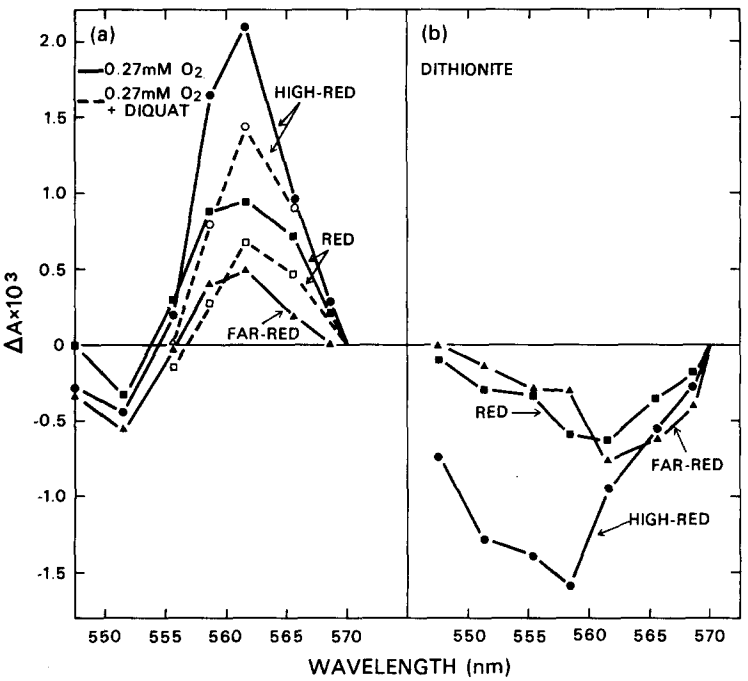

Fig. 10. Light minus dark difference spectra of developing pea plastids, isolated from dark-grown seedlings which had been greened for 3 h. Reference wavelength, 570 nm. Temperature, 27 °C. (a) Solid lines, in the presence of 0.27 mM O_2; broken lines, in the presence of 0.27 mM O_2 and diquat. Actinic light as shown: high intensity red (Corning 2-64) 260 kerg · cm^{-2} · s^{-1}; red (Corning 2-64 + Balzer K_6) 140 kerg · cm^{-2} · s^{-1}; far-red (Schott RG 715) 32 kerg · cm^{-2} · s^{-1}. Chlorophyll concentration, 22 μg/ml. (b) In the presence of approx. 5 mM dithionite.

TABLE VI

EFFECT OF DCMU ON CYTOCHROME *b*-563 PHOTOREDUCTION BY DEVELOPING PEA PLASTIDS

Plastids were isolated from seedlings greened for 3 h. Reference wavelength 570 nm. Temperature 27 °C.

Additions	Actinic light	Absorbance decrease at 563.5 nm (% of control)
10 μM DCMU	Corning 2-64	4
10 μM DCMU	Corning 2-64 +Balzer K6	10
10 μM DCMU +ascorbate/Cl_2Ind	Corning 2-64	60
10 μM DCMU +ascorbate/Cl_2Ind	Corning 2-64 +Balzer K6	93
10 μM DBMIB	Corning 2-64	25
10 μM DBMIB	Corning 2-64 +Balzer K6	34

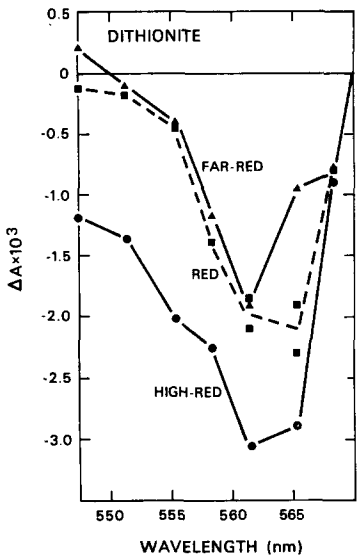

Fig. 11. Light minus dark difference spectrum of developing (3 h) pea plastids at 0 °C in the presence of approx. 5 mM dithionite. Reference wavelength, 570 nm. Actinic light intensities as for Fig. 10. Chlorophyll concentration, 18.7 μg/ml.

was observed in the presence of dithionite with high actinic light, both at room temperature and at 0 °C.

DISCUSSION

The response of intact CO_2-fixing chloroplasts to oxidants and reductants appears to be governed by the permeability of the chloroplast outer envelope. Previous studies [24, 28] have indicated that ferricyanide is not photoreduced by intact chloroplasts, and this is supported in the present work by (a) the lack of oxidation of cytochrome *f* and cytochrome b-559_{HP} by ferricyanide and (b) the variable fluorescence and high steady state level of fluorescence in the presence of ferricyanide. Determination of the extents of oxidation of cytochrome *f* and cytochrome b-559_{HP} in a preparation of chloroplasts before and after removal of the outer envelopes thus appears to be a convenient method for determining the degree of intactness of a chloroplast preparation.

The considerably lower rates of reduction of the low potential cytochromes (cytochrome b-563 and cytochrome b-559_{LP}) by dithionite, particularly at 0 °C, suggests that the chloroplast outer envelope is a permeability barrier to this reductant. The observation of variable fluorescence from intact chloroplasts at 0 °C in the presence of dithionite supports this view. It is known [25] that the level of fluorescence from chloroplasts is influenced by the redox state of the quencher Q (Fig. 5). With swollen or broken chloroplasts, Q is reduced by dithionite resulting in a high fluorescence level immediately on illumination of the chloroplasts, and therefore a lack of variable fluorescence. Hydroquinone and reduced dichloro-

phenolindophenol readily penetrate into intact chloroplasts, in contrast to ferr cyanide, ascorbate and dithionite.

Our cytochrome determinations on intact spinach chloroplasts show a high ratio of total cytochrome *b*/cytochrome *f* (5.2) than reported previously [19] f swollen spinach chloroplasts (3.6). The present values are more in agreement wi Plesnicar and Bendall's [4] figures for the cytochromes of barley chloroplasts (cyt chrome $b/f = 5.2$). Cytochrome *b*-563 is the most abundant cytochrome in inta spinach chloroplasts, and its concentration is double that of cytochrome *f* or cyt chrome b-559_{LP} and 50 % more than that of cytochrome b-559_{HP}.

Many investigators [7, 8, 10–15] have suggested that cytochrome *b*-563 ma function on a cyclic pathway around Photosystem I. Our data, both with inta spinach chloroplasts and developing pea plastids are consistent with this proposa It is difficult, however, to establish a definitive role for cytochrome *b*-563 in phot synthetic electron transport since the magnitude of the light-induced absorbanc changes attributable to this cytochrome are generally small and account for only small fraction of the cytochrome *b*-563 in the chloroplast. The small extents of th light-induced absorbance changes of cytochrome *b*-563 may be a consequence c its cyclic role in electron transport with the rates of oxidation and reduction bein nearly balanced.

Fig. 5 is a scheme for photosynthetic electron transport, showing cytochrom *b*-563 on a cyclic pathway around Photosystem I. The direction of flow aroun the cycle is dependent on the redox states of cytochrome *b*-563 and the componen Z and X, where the noncyclic and cyclic pathways diverge or converge. Our wor with intact chloroplasts suggests that cyclic flow is quite sensitive to the relati poising of the redox levels of cytochrome *b*-563 and the components of the noncycli pathway. Knaff [13] and Böhme and Cramer [11] have reported previously tha cytochrome *b*-563 is photooxidized in broken chloroplasts if the ambient potential i low enough to reduce cytochrome *b*-563 in the dark prior to illumination. With inta chloroplasts at 0 °C, dithionite penetrates very slowly but the anaerobic condition created by dithionite (or glucose plus glucose oxidase) are sufficient to cause som reduction of cytochrome *b*-563 in the dark.

Two observations require some comment: (1) the red/far-red antagonisr of the redox changes of cytochrome *b*-563 in intact chloroplasts made anaerobi with dithionite at 0 °C and (2) the DCMU inhibition of cytochrome *b*-563 reductio by red light at 0 °C in the presence of 3-phosphoglycerate. We do not conclud from these observations that cytochrome *b*-563 functions on the noncyclic pathwa between the photosystems, as well as on a cyclic pathway around Photosystem I DCMU will influence the redox levels of the electron carriers between the two phot systems. The redox state of these carriers would be expected to influence the cycli flow of electrons around the cytochrome *b*-563 pathway. If the carriers are largel reduced, electron flow from photoreduced cytochrome *b*-563 around the cycl would be inhibited, compared with the situation when the electron carriers are mor oxidized. The red/far-red antagonism can be explained in a similar way. The carrier between the photosystems will be more reduced in red than in far-red light and thi will cause a more reduced level of cytochrome *b*-563 in red light. The lack of inhibi tion by DBMIB of cytochrome *b*-563 photooxidation suggests that plastoquinon

is not a component of the cyclic pathway around Photosystem I, which contains cytochrome *b*-563.

The extent of the steady-state photoreduction of cytochrome *b*-563 in the developing pea plastids was considerably greater than in the intact spinach chloroplasts. This might suggest a rate-limiting step in the cycle on the oxidizing side of cytochrome *b*-563 in the immature plastids. Nonetheless, the photoreduction of cytochrome *b*-563 is strongly inhibited by DCMU and it could be restored by ascorbate plus Cl_2Ind. This suggests that the photoreduction of cytochrome *b*-563 in the developing pea plastids is also influenced by the redox states of the carriers between the photosystems.

The quantum yield measurements confirm previous work that cytochrome *f* oxidation is driven by Photosystem I. Photooxidation of cytochrome *b*-563 is also driven by Photosystem I with a quantum efficiency comparable to that for cytochrome *f*. The very low quantum yield for cytochrome *b*-563 photoreduction in intact spinach chloroplasts is consistent with the low steady-state levels of cytochrome *b*-563 reduction, and indicates that photoreduction in air is much less efficient than photooxidation under anaerobic conditions.

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