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PHOTOREACTIONS OF CYTOCHROME *b*-559 AND CYCLIC ELECTRON FLOW IN PHOTOSYSTEM II OF INTACT CHLOROPLASTS

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Summary

The high potential cytochrome *b*-559 of intact spinach chloroplasts was photooxidized by red light with a high quantum efficiency and by far-red light with a very low quantum efficiency, when electron flow from water to Photosystem II was inhibited by a carbonyl cyanide phenylhydrazone (FCCP or CCCP). Dithiothreitol, which reacts with FCCP or CCCP, reversed the photooxidation of cytochrome *b*-559 and restored the capability of the chloroplasts to photoreduce CO_2 showing that the FCCP/CCCP effects were reversible. The quantum efficiency of cytochrome *b*-559 photooxidation by red or far-red light in the presence of FCCP was increased by 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone which blocks oxidation of reduced plastoquinone by Photosystem I. When the inhibition of water oxidation by FCCP or CCCP was decreased by increased light intensities, previously photooxidized cytochrome *b*-559 was reduced. Red light was much more effective in photoreducing oxidized high potential cytochrome *b*-559 than far-red light. The red/far-red antagonism in the redox state of cytochrome *b*-559 is a consequence of the different sensitivity of the cytochrome to red and far-red light and does not indicate that the cytochrome is in the main path of electrons from water to NADP. Rather, cytochrome *b*-559 acts as a carrier of electrons in a cyclic path around Photosystem II. The redox state of the cytochrome was shifted to the oxidized side when electron transport from water became rate-limiting, while oxidation of water and reduction of plastoquinone resulted in its shifting to the reduced side.

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

Introduction

The chloroplast thylakoid membrane contains two *b* cytochromes with α-bands at 559 nm, distinguishable by their different midpoint potentials [1,2]. High potential cytochrome *b*-559 (cytochrome b-559_{HP}) with a midpoint potential of about +0.37 V at pH 7 [2] accounts for some 60% of the total cytochrome *b*-559 in intact chloroplasts capable of high rates of CO_2 reduction [3]. Low potential cytochrome *b*-559 (cytochrome b-559_{LP}) has a midpoint potential of about +0.065 V and unlike the high potential form is in the oxidized state in chloroplasts. Cytochrome b-559_{HP} is closely associated with Photosystem II [4,5], whereas cytochrome b-559_{LP} is found in Photosystem I particles, in association with cytochrome *b*-563 and cytochrome *f* [6,7]. Cytochrome b-559_{HP} in chloroplasts is converted to a form of lower potential by aging, mild heating or incubation with detergent [8], but this form should be distinguished from cytochrome b-559_{LP} found in freshly prepared, intact CO_2-fixing chloroplasts. Light-induced redox changes have not been established with cytochrome b-559_{LP}, and its function in chloroplasts is unknown. The role of cytochrome b-559_{HP} is still unclear even though it has been studied quite extensively. Thus, it has been suggested that it functions in the main electron transport chain between the two photoacts of Photosystems I and II [9–12], in the water-splitting sequence of reactions [13,14], on a side pathway from Photosystem II with a possible additional link to Photosystem I [15,16] or in a cycle around Photosystem II [17–19].

The main reason for the uncertainty about the role of cytochrome *b*-559 is the lack of light-induced redox changes at room temperature in fresh preparations of higher plant chloroplasts [5,16,20]. Photooxidation of cytochrome *b*-559 is observed at room temperature in the presence of CCCP, an uncoupler of phosphorylation [10,16,20] or after treatment of chloroplasts with tris-(hydroxymethyl)-aminomethane [5], but there are conflicting views as to whether the photooxidation is driven by Photosystems I or II. There is also disagreement on the photoreduction of cytochrome *b*-559 by Photosystem II. Cramer et al. [21] and Horton and Cramer [22,23] suggested that CCCP or protonation converts cytochrome b-559_{HP} to a form of lower potential, which then functions between the systems. At liquid nitrogen temperature, a photooxidation of cytochrome b-559_{HP} driven by Photosystem II is observed with untreated chloroplasts [5,15,24,25].

In this communication, we report experiments with intact CO_2-fixing chloroplasts and conclude that cytochrome b-559_{HP} is involved in cyclic electron transport around Photosystem II. Light-dependent redox changes similar to those seen in intact chloroplasts can also be observed after the chloroplast envelope has been ruptured and soluble chloroplast components have been released.

Materials and Methods

Intact chloroplasts, capable of high rates of CO_2 fixation (100–200 μmol CO_2-dependent O_2 evolution · mg^{-1} chlorophyll · h^{-1}) were isolated from field-grown or hydroponically grown spinach, as described previously [26]. The

reaction medium for measuring cytochrome redox changes contained 300 mM sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM ethylenediamine tetraacetate, 0.5 mM potassium phosphate, 10 mM NaCl and 50 mM Hepes buffer, pH 7.6. Catalase (2000 units · ml^{-1}) was usually added.

Light-induced redox changes of cytochromes were measured in an Aminco Chance dual wavelength spectrophotometer, equipped with a side illumination system. Monochromatic actinic light was produced by passing parallel light through the following filter combinations: 80 mm water, 3 mm RG 630 cut off (Schott, Mainz), 1 mm Calflex C and interference filters 651 nm, 664 nm, 674 nm, 710 nm or 720 nm (half bandwidth 12 nm, from Balzers, Liechtenstein). Red light of broad bandwidth was obtained by omitting the interference filter from the above combination of filters, or substituting a broad-band interference filter (K_6, Balzer) in place of the narrow band interference filter. Light intensities were measured with a silicon photodiode. Other methods, including those used to measure quantum requirements, are described elsewhere [3,26].

Results

Intact chloroplasts can photoreduce CO_2 or oxaloacetate. In contrast to CO_2 reduction, there is no requirement for ATP, when oxaloacetate is the electron acceptor. Fig. 1 shows light-dependent absorbance changes of intact chloroplasts at 561 nm in the presence of 5 μM FCCP and 1 mM oxaloacetate.

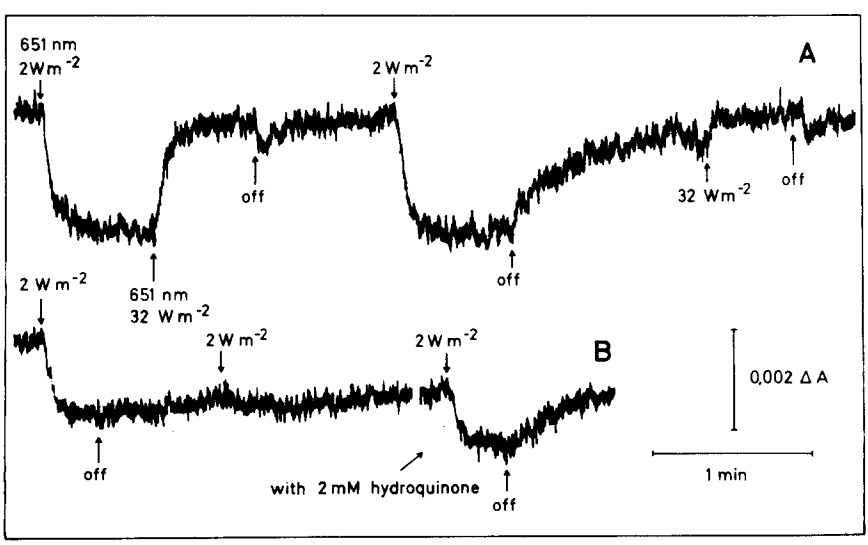

Fig. 1. (A) Oxidation of cytochrome *b*-559 by low intensity 651 nm light (2 W · m^{-2}) and reduction under high intensity illumination (32 W · m^{-2}) as seen from absorption changes at 561 nm (reference wavelength 570 nm). An absorption decrease denotes oxidation, an increase reduction. The absorption decrease seen when the high intensity 651 nm beam was turned off was caused by dark oxidation of photoreduced cytochrome *b*-563. The second illumination with high intensity light caused only cytochrome *b*-563 reduction. (B) Irreversible photooxidation of cytochrome *b*-559 in the absence and reversibility of the reaction in the presence of 2 mM hydroquinone. Measurements at 561 nm. The reaction medium contained chloroplasts with 75 μg chlorophyll · ml^{-1}, 5 μM FCCP and 1 mM oxaloacetate as electron acceptor.

Before the addition of FCCP, the chloroplasts had reduced CO_2 under light-saturated conditions at a rate of 187 μmol · mg^{-1} chlorophyll · h^{-1}. This reaction was completely inhibited by FCCP which acts as uncoupler. After FCCP was added, low intensity red monochromatic light (651 nm, 2 W · m^{-2}) caused a large decrease in absorbance at 561 nm, indicative of the oxidation of a cytochrome. A subsequent increase in the light intensity at 651 nm to 32 W · m^{-2} reversed the reaction. Darkening after high intensity light caused a small, rapid decrease in absorbance, followed by a slow absorbance increase. Addition of 2 μM DBMIB to the reaction medium did not appreciably alter the pattern of redox absorption changes seen in Fig. 1A (not shown). A much lower concentration, 0.7 μM DBMIB, was sufficient to completely inhibit oxygen evolution by intact chloroplasts. The cytochromes undergoing redox changes at 561 nm can be identified from the difference spectra in Fig. 2. The maximum of the negative absorption change caused by low intensity 651 nm light was at 558 nm, both in the presence or absence of DBMIB. The absorption increase caused by higher intensity 651 nm light was at a wavelength slightly higher than 559 nm. It is concluded that cytochrome *b*-559 accounts for a large part of the absorbance changes seen in Fig. 1A. Thus cytochrome *b*-559 is photo-oxidized in low intensity red light and re-reduced as the light intensity is

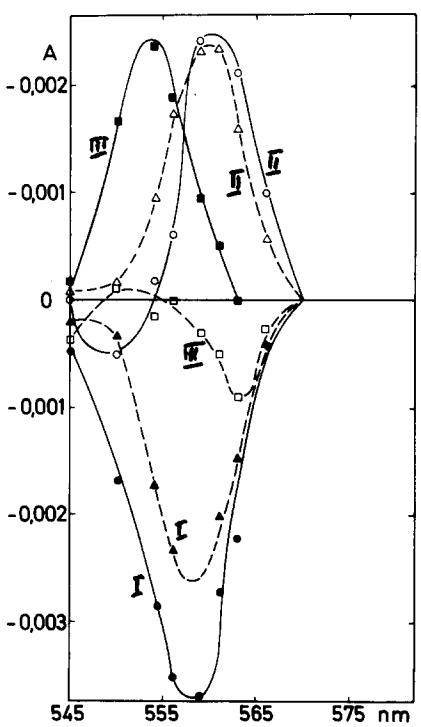

Fig. 2. Difference spectra of absorption changes caused by low and high intensity illumination with 651 nm light and by darkening. Conditions as in Fig. 1, but with 10 μM FCCP. Sequence of illuminations: 30 s 2 W · m^{-2} (low), 30 s 32 W · m^{-2} (high), dark. Measurements were taken after 30 s in low light (I), after 10 s in high light (II), and after 8 s in the dark (III): ------, without DBMIB; ———, with 3 μM DBMIB.

increased, and neither redox change is inhibited by 3 μM DBMIB. The small absorbance decrease which was seen on turning off the 32 W · m^{-2} light in the absence of DBMIB light showed a maximum at 563 nm, indicative of the dark oxidation of photoreduced cytochrome *b*-563. In the presence of DBMIB there was a rapid absorbance increase at 554 nm in the dark, (Fig. 2, curve III) due to a fast reduction of photooxidized cytochrome *f*. A rapid dark reduction of cytochrome *f* in DBMIB treated chloroplasts has been reported previously [27].

The 3-wavelength equation of Heber et al. [3] was used to calculate the contribution of cytochrome *b*-559 and cytochrome *f* to the absorbance changes displayed in Fig. 2. The values, which should be regarded as semiquantitative [3] are given in Table I. About one-half of cytochrome *b*-559_{HP} was photooxidized by low intensity 651 nm light, and there was no inhibition by DBMIB. In fact, DBMIB caused an increase in the extent of photooxidation. Increasing the light intensity of the 651 nm beam from 2 W · m^{-2} to 32 W · m^{-2} caused a very considerable re-reduction of the photooxidized cytochrome *b*-559_{HP}. A small percentage of cytochrome *b*-563 which was in the oxidized state in the dark was also photoreduced by the higher light intensity. Cytochrome *f* was partly oxidized (20%) by the low intensity beam, and the extent of photooxidation was doubled by the presence of DBMIB.

Since cytochrome *b*-559_{HP} is the only *b* cytochrome which is in the reduced state in intact chloroplasts in the dark [3], it is concluded that cytochrome *b*-559_{HP} is photooxidized by low intensity red light and re-reduced by sequential illumination with high intensity red light. This conclusion is supported by absorbance changes shown in Fig. 1B. In the absence of an exogenous reducing agent, cytochrome *b*-559_{HP} remained oxidized in the dark after illumination with low intensity red light or with far-red light sufficient to exhaust endogenous reductants. In the experiment of Fig. 1B, photooxidation of cytochrome *b*-559_{HP} was observed at the first illumination with low intensity red light, but a second illumination was ineffective, because little re-reduction of cytochrome *b*-559_{HP} occurred in the dark. Addition of 2 mM hydroquinone, which reduces

TABLE I

LIGHT-INDUCED REDOX CHANGES OF CYTOCHROMES

Calculated from difference spectra of Fig. 2.

Illumination conditions (651 nm light)	DBMIB	Extent of absorbance change (mol cytochrome/100 mol chlorophyll)		% of cytochrome reacting	
		cyt *b*-559_{HP}	cyt *f*	cyt *b*-559_{HP}	cyt *f*
Dark → 2 W · m^{-2}	—	1.4 ox *	0.4 ox	45	20
	+	1.8 ox	0.8 ox	55	40
2 W · m^{-2} → 32 W · m^{-2}	—	1.3 red	—	40	—
	+	1.4 red	0.4 ox	45	20
32 W · m^{-2} → dark	—	0.1 red	0.2 ox		
	+	0.4 red	1.2 red		

* ox, oxidized; red, reduced.

cytochrome b-559$_{HP}$ but not cytochrome b-559$_{LP}$, permitted a reversible photooxidation of cytochrome b-559$_{HP}$ at each successive illumination with low intensity red light.

The experiment reported in Fig. 3 shows that the cytochrome reduced by high intensity red light is the one which is oxidized by low intensity red light. For this experiment, ascorbate and cysteine were omitted from the medium used to isolate the intact chloroplasts. Chloroplasts in reaction medium with 5 μM CCCP were repeatedly preilluminated with 651 nm light of intensity 3 W · m^{-2} and then given a long dark period to exhaust endogenous reductants and to oxidize any cytochrome b-559$_{LP}$ which might have been photo-reduced. Illumination with low intensity red light (Fig. 3A) caused an absorbance decrease at 651 nm, which was only partially reversed in the subsequent dark period. The difference spectrum (Fig. 3B) indicates photooxidation of cytochrome b-559$_{HP}$ (0.8 moles/1000 mol chlorophyll) and some cytochrome f. After a dark period of 1 min the chloroplasts were illuminated with high intensity 651 nm light (46 W · m^{-2}). This caused an increase in absorbance at 561 nm, and the difference spectrum (Fig. 3B, curve b) showed a peak at about 559 nm. Calculation by the 3-wavelength equations revealed the reduction (per 1000 mol of chlorophyll) of 0.8 mol of cytochrome b-559, 0.4 mol cyto-

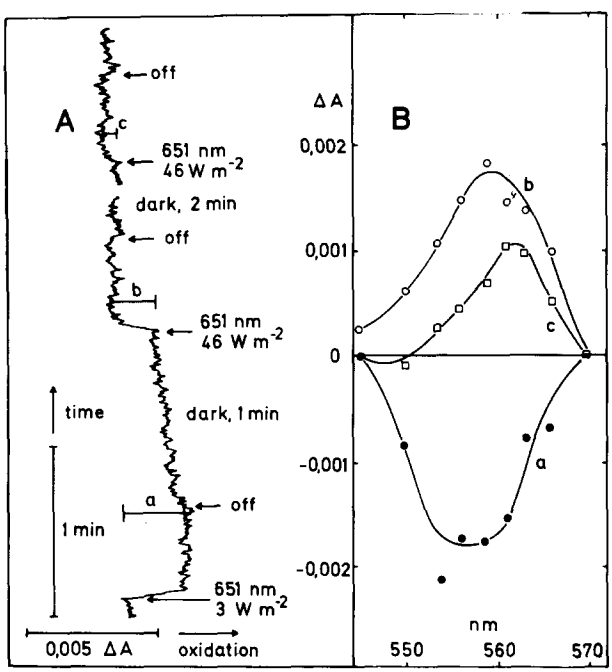

Fig. 3. (A) Oxidation of cytochrome b-559 in intact chloroplasts by low intensity 651 nm light, as seen by a decrease in absorption at 561 μm. The photooxidation was not reversed in the dark in the intact chloroplasts which did not contain endogenous reductant, but it was reversed by high intensity 651 nm light. A second illumination with high intensity 651 nm light reduced only cytochrome b-563. Conditions as in Fig. 1. (B) Difference spectra of absorption changes in (A). Schedule: 30 s low intensity 651 μm light (3 W · m^{-2}), 60 s dark, 30 s high intensity 651 nm light (46 W · m^{-2}), 120 s dark, 20 s high intensity 651 nm light, dark. Measurements were taken after 30 s in low intensity light (curve a) and after 10 s in high intensity light, first illumination (curve b), second illumination (curve c).

chrome *b*-563 and some cytochrome *f*. The cytochrome *b*-559, which was photoreduced by high intensity light was the same high potential cytochrome which previously had been photooxidized by low intensity light. This was established by allowing the chloroplasts to remain in darkness for 2 min and then illuminating them again with high intensity light. Any photoreduced cytochrome $b\text{-}559_{LP}$ would have been reoxidized in the two minute dark period, since this cytochrome is autoxidizable [1—3]. The difference spectrum from the second illumination (Fig. 3B, curve c) shows a peak at 563 nm, due mainly to the reduction of cytochrome *b*-563.

Fig. 4 compares the effects of red and far-red light on the photooxidation of cytochrome $b\text{-}559_{HP}$ in intact chloroplasts. The light intensities were selected so that the chloroplasts absorbed slightly more quanta from the far-red beam than from the red beam. A suspension of intact chloroplasts in a large Ulbricht sphere at a concentration of 75 μg chlorophyll · ml^{-1} absorbed 60% of 710 nm light and 93% of 651 nm light. The wavelength maximum in the difference spectrum from far-red illumination was about 2 nm lower (at 557 nm) than that from red illumination. In the presence of 3 μM DBMIB, there was a further shift in the maximum of the difference spectrum, induced by far-red light, to a shorter wavelength (555 nm), and the amplitude was greater. The amounts of cytochrome $b\text{-}559_{HP}$ and cytochrome *f* photooxidized in the steady state are shown in Table II. Although the amplitude of cytochrome $b\text{-}559_{HP}$ oxidation is only slightly less in 710 nm light than in 651 nm light, the rate of photooxidation was slower in the far-red beam. DBMIB had only a small effect on the extent of photooxidation of cytochrome $b\text{-}559_{HP}$ in far-red light, but it increased cytochrome *f* photooxidation. The very poor inhibition by DBMIB of cytochrome $b\text{-}559_{HP}$ photooxidation driven by far-red light with intact CO_2 fixing chloroplasts does not agree with earlier reports with other chloroplast

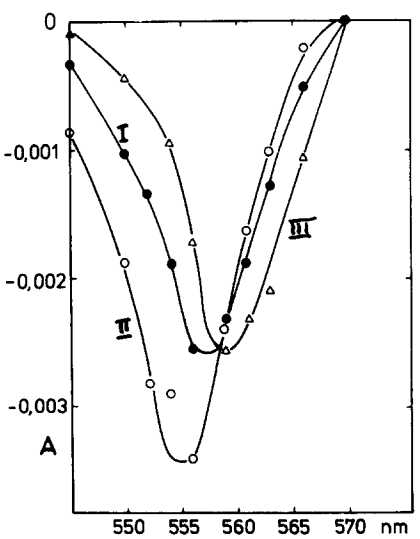

Fig. 4. Dark/light difference spectra of absorption changes caused by red and far-red light. Curves I and II; 710 nm light of intensity 1.2 W · m^{-2}. Curve III; 651 nm light of intensity 0.6 W · m^{-2}. DBMIB (3 μM) was included in the reaction medium for curve II.

TABLE II

CYTOCHROME REDOX CHANGES IN RED AND FAR-RED LIGHT

Calculated from difference spectra shown in Fig. 5.

Actinic light	DBMIB (3 μM)	Extent of cytochrome oxidation (mol cytochrome/100 mol chlorophyll)	
		Cytochrome b-559$_{HP}$	Cytochrome f
710 nm, 1.2 W · m^{-2}	—	1.2	0.5
	+	1.1	1.2
651 nm, 0.6 W · m^{-2}	—	1.4	—

preparations [21,27,28]. Fig. 5 shows the influence of 3 μM and 10 μM DBMIB on the photooxidation of cytochrome b-559$_{HP}$ in low intensity red (6 W · m^{-2}) and medium intensity far-red light (21 W · m^{-2}). There is some inhibition of both the far-red and red-driven oxidation of cytochrome b-559$_{HP}$ with 10 μM DBMIB and the subsequent reductions with 651 nm appear faster than in the control. However 10 μM DBMIB is considerably in excess of the concentration (0.7 μM) required to effectively inhibit electron flow.

The effect of light intensity on the extent of the absorbance change at 561 nm are shown in Fig. 6. The absorbance changes at 561 nm mainly monitor the redox state of cytochrome b-559$_{HP}$, although at higher light inten-

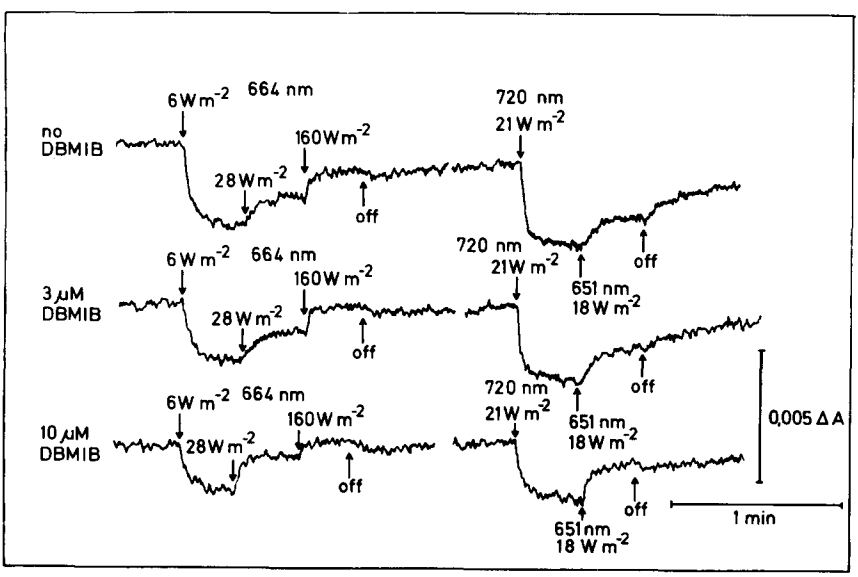

Fig. 5. Effect of 3 μM DBMIB and 10 μM DBMIB on the photoxidation of cytochrome b-559$_{HP}$ by low intensity red or medium intensity far-red light and on the photoreduction of cytochrome b-559$_{HP}$ by high intensity red light. The far-red light was turned off when the red beam was turned on. The extents of the absorption changes were decreased by 10 μM DBMIB, but the essential features of oxidation and reduction were similar with and without DBMIB. Measurements were taken at 561 μM, with a reference wavelength of 570 nm. Conditions as for Fig. 1, but 2.5 mM ascorbate also was present.

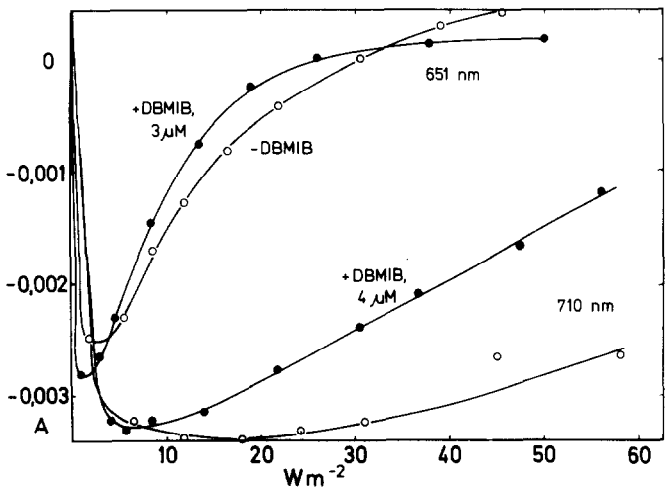

Fig. 6. The effect of the intensity of 651 or 710 nm light on the extent of the absorbance change at 561 nm. Conditions as in Fig. 1.

sities, there is some contribution from the reduction of cytochrome *b*-563 (Table I) and in the presence of DBMIB, the contribution of cytochrome *f* to the absorbance change at 561 nm is not negligible. Low intensities of 651 nm light were more effective in bringing about photooxidation of cytochrome b-559_{HP} than the same intensities of 710 nm light. As the intensity of the 651 nm beam is increased above 2 $W \cdot m^{-2}$, there is a progressive decline in the amplitude of photooxidation of cytochrome b-559_{HP}. Between 4 $W \cdot m^2$ and 30 $W \cdot m^{-2}$ of 651 nm light, cytochrome b-559_{HP} was slightly less oxidized in the presence of 3 μM DBMIB. At a high light intensity (>30 $W \cdot m^{-2}$) the increase in absorbance at 561 reflects the reduction of cytochrome *b*-563, and this is inhibited by DBMIB [3]. As with 651 nm light, higher intensities of 710 nm light were less effective in causing photooxidation of cytochrome b-559_{HP} than low intensity light. The reductive shift caused by DBMIB in the redox state of cytochrome b-559_{HP} at high light intensities of 651 nm light is also observed in 710 nm light at intensities above 10 $W \cdot m^{-2}$.

It was necessary to establish that FCCP and DBMIB remained effective after addition to the chloroplasts. FCCP reacts with SH-groups [29] and thereby loses its uncoupling properties. Illuminated chloroplasts generate thiol groups [30], which slowly inactivate FCCP. This explains why oxygen evolution slowly reappears in CO_2 fixing chloroplasts inhibited with low concentrations of FCCP. DBMIB is also capable of reacting with SH-groups [31]. A slow inactivation of DBMIB was observed in our experiments. Intact chloroplasts which had been illuminated for 30 min in the presence of oxaloacetate as electron acceptor and 3 μM DBMIB showed 30% of the O_2 evolving capacity of control chloroplasts in the absence of DBMIB. However, in our work the concentrations of FCCP and DBMIB were sufficient to ensure complete uncoupling of phosphorylation (FCCP) and inhibition of electron flow (DBMIB) during the time of the individual experiments.

It was proposed in an earlier study [16] that the photooxidation of cyto-

chrome b-559$_{HP}$ by Photosystem II in the presence of FCCP may be due to an inhibition by FCCP of electron flow between water and Photosystem II [32]. Anderson et al. [28] reported a rather small (25%) inhibition of electron flow from water to 2,3',6-trichlorophenolindophenol by 1.5 μM FCCP. Fig. 7 shows that 1.5 μM CCCP (CCCP is a less potent uncoupler than FCCP) caused maximum photooxidation of cytochrome b-559$_{HP}$ by 651 μm light or a broad band of red light. There is a striking correlation between the extent of cytochrome b-559$_{HP}$ oxidation with increasing concentrations of CCCP and the decrease in the 518 nm electrochromic shift. This result would suggest a close correlation between the uncoupling action of CCCP and cytochrome b-559$_{HP}$ photooxidation.

In an attempt to distinguish between uncoupling and inhibition of electron flow as a possible cause of the photooxidation of cytochrome b-559$_{HP}$ by Photosystem II, we have examined the effect of FCCP on the quantum requirement of oxaloacetate reduction by intact chloroplasts (Fig. 8). In the absence of FCCP, the quantum requirement of oxaloacetate reduction increased with

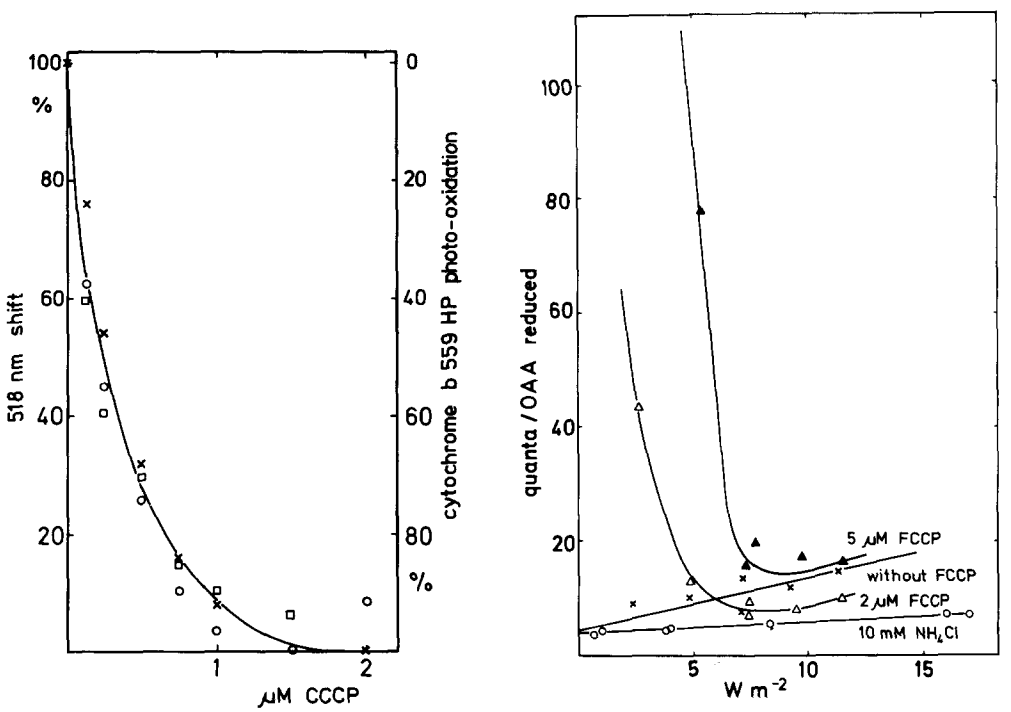

Fig. 7. Effect of CCCP concentration on the photooxidation of cytochrome b-559$_{HP}$ and the light-induced absorbance increase at 518 nm (electrochromic shift) in intact chloroplasts: Absorption changes are normalized to permit comparison. ○, cytochrome b-559$_{HP}$ oxidation in 651 nm light of intensity 6 W · m^{-2}; □, 518 nm absorbance change in 651 nm light of intensity 6 W · m^{-2}. X, 518 nm absorbance change in a broad band of red light of intensity approx. 300 W · m^{-2}.

Fig. 8. Quantum requirement of oxaloacetate reducation by intact chloroplasts as a function of the intensity of 674 nm light. Chloroplasts at a concentration of 66 μg chlorophyll · ml^{-1} were illuminated in a CO_2-free medium in the presence of 2 mM oxaloacetate. Other conditions as indicated on curves. Oxygen evolution served as a measured of oxaloacetate reduction.

light intensity. This was not due to a limitation of the reaction by the malic dehydrogenase content of the chloroplasts. It appears that photosynthetic control by the intrathylakoid pH was responsible for the increase in quantum requirement, since it was abolished by the addition of NH_4Cl as uncoupler. In the presence of NH_4Cl, 4 quanta of 674 nm light were required for the reduction of one molecule of oxaloacetate to malate. When FCCP was added as uncoupler, however, the quantum requirement of oxaloacetate reduction increased very markedly as the light intensity was reduced. Low light intensities were now completely ineffective in driving the reduction of oxaloacetate. When the FCCP concentration was increased, from 2 μM to 5 μM, more light was required to reduce oxaloacetate with a quantum efficiency comparable to that of samples without FCCP. It is clear from these results that water oxidation is inhibited at low intensities of red light, conditions under which photooxidation of cytochrome b-559_{HP} is high. As the light intensity is increased, the quantum efficiency of oxygen evolution rises, and the redox state of cytochrome b-559_{HP} shifts towards reduction.

To assess the relative effectiveness of red and far-red light in oxidizing cytochrome b-559_{HP}, we have determined the quantum requirements for cytochrome b-559_{HP} photooxidation. Fig. 9 shows the rate of photooxidation of cytochrome b-559_{HP} as a function of intensity of red (651 nm) and far-red (710 nm) light. Far-red light which excites mainly Photosystem I was much less effective than red light, even after allowing for the lower absorption of far-red light by chloroplasts. Quantum requirements are shown in Tables III and IV. Red light was reasonably efficient in photooxidizing cytochrome b-559_{HP}; the average quantum requirement was 6.7. Far-red light (710 nm or 720 nm) was much less efficient since 36 quanta of 710 nm light and 65 quanta of 720 nm were required to oxidize one molecule of cytochrome b-559_{HP}. In contrast, the photooxidation of cytochrome f by far-red light under similar conditions, except that DCMU was present, required only 4 quanta per molecule [3]. The

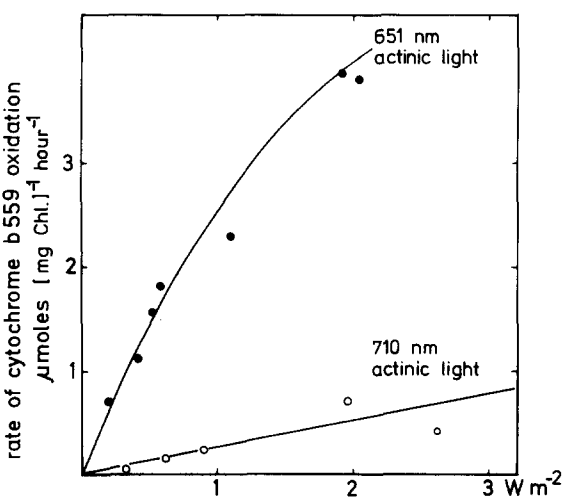

Fig. 9. Rate of cytochrome b-559_{HP} photooxidation as a function of the intensity of 651 or 710 nm light. Conditions as for Fig. 1.

TABLE III

QUANTUM REQUIREMENTS FOR OXIDATION OF CYTOCHROME b-559$_{HP}$

The reaction mixture contained 5 μM FCCP and 1 mM oxaloacetate. In some of the experiments 2 mM ascorbate was also present. Chlorophyll concentration, 75 $\mu g \cdot ml^{-1}$. Temperature, 22°C.

Wavelength of actinic light (nm)	No. of experiments	Quantum requirement	
		(quanta absorbed/molecule cytochrome oxidized)	Standard deviation
651	18	6.7	1.9
710	15	36	16.2
720	21	65	21.4

values obtained should not be regarded as minimum ones since any cyclic oxidoreductions would be expected to increase the quantum requirements. Table IV indicates that the efficiency of cytochrome b-559$_{HP}$ oxidation as measured by the absorption change at 561 nm was increased by 3 μM DBMIB both in red and far-red light. Cytochrome f photoxidation may have contributed to the result, although cytochrome f absorbs poorly at 561 μm.

There is the possibility that the photooxidation of cytochrome b-559$_{HP}$ by far-red light was driven not by Photosystem I, but by a small absorption of far-red light by Photosystem II. The absorption of far-red light by Photosystem II was determined from measurements of oxygen evolution, either with CO_2 or, after uncoupling by NH_4Cl, with oxaloacetate as electron acceptor. The intensities of light (651 nm, 710 nm or 720 nm) were low enough to ensure proportionality between the rate of oxygen evolution and light intensity. On the basis of absorbed quanta, 710 nm light gave 23% and 720 nm light 6% of the oxygen evolution observed under 651 nm light. It would appear, therefore, that Photosystem I is even less efficient in photooxidizing cytochrome b-559 than is apparent from the quantum requirements in Table IV.

TABLE IV

EFFECT OF DBMIB ON QUANTUM REQUIREMENTS FOR OXIDATION OF CYTOCHROME b-559$_{HP}$

The reaction mixture contained 5 μM FCCP, 1 mM oxaloacetate and 2 mM ascorbate. Data are uncorrected for any contribution of cytochrome f to the absorbance changes at 561 nm.

Experiment No.	Wavelength of actinic light	Quantum requirements (quanta absorbed/molecule cytochrome oxidized)	
		Minus DBMIB	+ 3 μM DBMIB
1	651	7.8	3.8
2	651	5.7	3.8
3	651	5.9	3.6
4	651	8.3 *	4.5
1	720	45	24
2	720	46	24
3	720	66	28
4	720	83 *	36

* Ascorbate omitted.

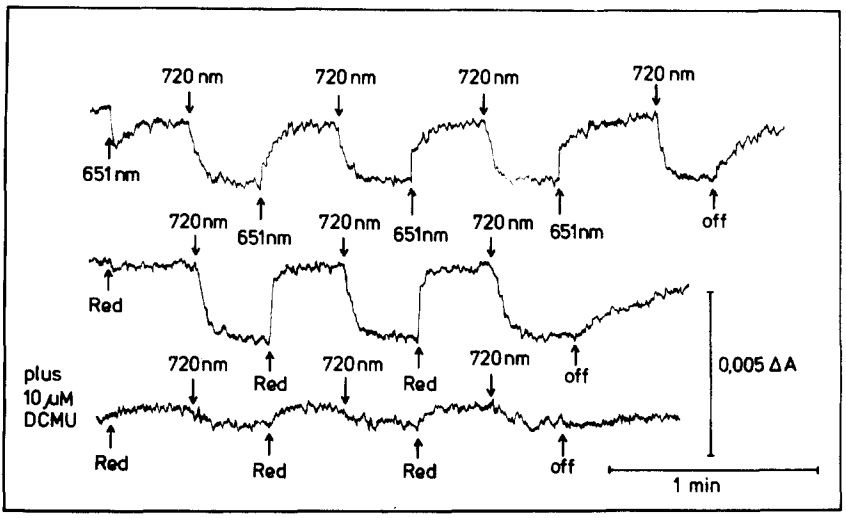

Fig. 10. Apparent red/far-red antagonism in the redox state of cytochrome b-559$_{HP}$ as measured from absorption changes at 561 nm in the presence of 3 μM DBMIB. The addition of 10 μM DCMU inhibited the redox changes due to cytochrome b-559$_{HP}$. Conditions as for Fig. 1, except for presence of DBMIB. Addition of DCMU as indicated. Light intensities: 651 nm, 22 W · m^{-2}; 720 nm, 26 W · m^{-2}; red (broad band of short wavelength red light), 160 W · m^{-2}.

The red/far-red antagonism shown in the redox changes of cytochrome b-559$_{HP}$ (Fig. 10) resembles the well-known behaviour of cytochrome f, which provided evidence for the concept of two photosystems operating in series. However, 3 μM DBMIB which inhibits electron flow between the two photoacts was present in the experiments of Fig. 10, and the oxidation and reduction of cytochrome b-559$_{HP}$ cannot be explained by the antagonistic effects of Photosystems I and II. The oxidation of cytochrome b-559 whether driven by red or far-red light was inhibited by 10 μM DCMU. Difference spectra showed that the residual absorbance changes seen in the presence of DCMU were due to cytochrome f oxidation in red and far-red light and cytochrome b-563 reduction in red light.

Discussion

Cramer and Whitmarsh [33] have recently discussed evidence on the function of cytochrome b-559 in photosynthesis. An earlier proposal that the cytochrome is located in the main chain between Photosystems II and I was mainly based on the observation of antagonistic photoreduction and oxidation of the cytochrome by red and far-red light. We have now shown that such antagonistic responses are still observed when the main path of electrons is blocked by the plastoquinone antagonist DBMIB (Fig. 10). They are easily explained by the different sensitivity of the cytochrome to oxidation and reduction by red and far-red light as shown in Fig. 7. Recent experiments of Whitmarsh and Cramer [34] have also indicated that cytochrome b-559 is not an obligatory redox component of the main electron transport chain. The disagreement in the literature whether cytochrome b-559 is preferentially oxidized by Photosystems II

or I [16,18,21,27,28,35] can also be resolved. In the presence of FCCP, which inhibits water splitting much more at low than at high light intensities (Fig. 8 and Refs. 36, 37), the amplitude of photooxidation is larger under illumination with a high intensity of far-red light than with a corresponding intensity of red light (Fig. 6). This is a consequence of the photoreduction of cytochrome *b*-559 by red light of sufficiently high intensities. However, the quantum requirement data of Table III clearly show that Photosystem II is much more effective in oxidizing cytochrome *b*-559 than Photosystem I. Since the reactive cytochrome can be reduced by hydroquinone in the dark, it must be a high potential species. The low potential cytochrome *b*-559 known to exist in the oxidized state in intact chloroplasts in the dark [3] did not show a significant change in its redox state upon illumination (Fig. 3B), although plastoquinone with a similar redox potential is known to become reduced.

We concluded from the redox behaviour of cytochrome *b*-559$_{HP}$ that it is involved in cyclic electron transport around Photosystem II. The occurrence of such a cycle has been repeatedly suggested [17—19,38]. Oxidation and reduction of the same electron carrier by the same photosystem is direct evidence for a cyclic electron transport pathway. The fact that addition of a compound such as FCCP is necessary to demonstrate the pathway does not imply that it is a non-physiological phenomenon. The failure to observe much photooxidation of cytochrome *b*-559 in undisturbed chloroplasts shows only that water is an effective electron donor to Photosystem II. The redox state of cytochrome *b*-559 is shifted to the oxidized side, when photolysis of water is affected. The inhibition of water splitting by FCCP or CCCP is neither drastic nor irreversible. It can be overcome by simply raising the light intensity (Figs. 1 and 8) or by adding dithiothreitol. Oxidation of cytochrome *b*-559, when water oxidation is inhibited, suggests competition in electron donation to Photosystem II. Such competition is also apparent from low temperature experiments [33]. Since the maximum rate of water oxidation is limited, it is suggested that photooxidation of cytochrome *b*-559 (and its cyclic reduction) is a physiological reaction under conditions such as high light intensities, when the oxidation capacity of the chlorophyll system exceeds that of the water splitting machinery. Oxidation of cytochrome *b*-559 would then induce cyclic electron flow preventing photooxidative reactions of the strong oxidant generated by Photosystem II. Under high light the electron transport chain is largely reduced making it difficult to detect cyclic electron flow through Photosystem II in undisturbed chloroplasts. Electron transport through cytochrome *b*-559$_{HP}$ from Photosystem II to Photosystem I appears to be inefficient as shown by the low quantum yield of cytochrome *b*-559 oxidation by far-red light.

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