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KINETICS OF THE PHOTOREDUCTION OF CYTOCHROME *b*-559 BY PHOTOSYSTEM II IN CHLOROPLASTS

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SUMMARY

The kinetics of the photoreduction of cytochrome *b*-559 and plastoquinone were measured using well-coupled spinach chloroplasts. High potential (i.e. hydroquinone reducible) cytochrome *b*-559 was oxidized with low intensity far-red light in the presence of *N*-methyl phenazonium methosulfate or after preillumination with high intensity light. Using long flashes of red light, the half-reduction time of cytochrome *b*-559 was found to be 100 ± 10 ms, compared to 6–10 ms for the photoreduction of the plastoquinone pool. Light saturation of the photoreduction of cytochrome *b*-559 occurred at a light intensity less than one-third of the intensity necessary for the saturation of ferricyanide reduction under identical illumination conditions. The photoreduction of cytochrome *b*-559 was accelerated in the presence of dibromothymoquinone with a $t_{\frac{1}{2}} = 25$–35 ms. The addition of uncouplers, which caused a stimulatory effect on ferricyanide reduction under the same experimental conditions, resulted in a decrease in the rate of cytochrome *b*-559 reduction. The relatively slow photoreduction rate of cytochrome *b*-559 compared to the plastoquinone pool implies that electrons can be transferred efficiently from Photosystem II to plastoquinone without the involvement of cytochrome *b*-559 as an intermediate. These results indicate that it is unlikely that high potential cytochrome *b*-559 functions as an obligatory redox component in the main electron transport chain joining the two photosystems.

INTRODUCTION

Although there is a large amount of experimental evidence dealing with cytochrome *b*-559 its role in photosynthesis is still in question [1]. It has been proposed that it may function (a) as a redox component in the main electron transport chain joining the two photosystems [2, 3], (b) in a cycle around Photosystem II [4], or (c)

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (dibromothymoquinone); DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; PMS, *N*-methyl phenazonium methosulfate; S-13, 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanide.

in water splitting [5, 6]. In the present work we will be concerned primarily with the first of these hypotheses. Cytochrome *b*-559 has a relatively high potential component with a midpoint potential of 0.35–0.40 V [7], and it appears to be in close structural proximity with Photosystem II (8–10). The suggestion of a function in the main chain is based on observation of antagonistic oxidation by far-red light oxidation and red light reduction of a cytochrome with a reduced α-band maximum at 559–560 nm in chloroplast fragments of the green algae *Chlamydomonas reinhardi* [2] and in spinach chloroplasts [11]. In spinach chloroplasts the amplitude of the far-red oxidation of initially reduced cytochrome *b*-559 is small without some pretreatment. A larger amplitude of far-red oxidation can be observed in the presence of the uncoupler FCCP [11–14] or S-13 [15], in the presence of low concentrations of PMS [16], at low pH [17], or after preillumination with high intensity actinic light [17]. The latter three conditions are more physiological and allow experimentation using chloroplasts that are capable of coupled phosphorylation. Inhibition of the far-red light oxidation by a low concentration of DBMIB implies that the pathway of photooxidation utilizes at least part of the plastoquinone pool [14]. A pecularity of these more physiological conditions is that the amplitude of the oxidation induced by far-red light (732 nm) decreases as the light intensity is increased above 10^4 ergs · cm^{-2} · s^{-1} [16, 17]. The reason for this decline in oxidation efficiency is not known, but the consequence is that the rates of oxidation are necessarily slow.

One test of main chain function of a redox component is to compare the rates of oxidation-reduction with physiological turnover rates. With this in mind, and in an effort to better determine the relation of cytochrome *b*-559 to the known electron transport components between Photosystem II and Photosystem I, we have measured the kinetics of the reduction of high potential cytochrome *b*-559 and of plastoquinone by high intensity saturating flashes of red light. The relatively slow rate of cytochrome *b*-559 photoreduction compared to the plastoquinone reduction rate leads us to conclude that oxidized cytochrome *b*-559 is not an efficient acceptor of electrons from Photosystem II. It is therefore unlikely that high potential cytochrome *b*-559 functions as an obligatory redox component in the main electron transport chain.

MATERIALS AND METHODS

Chloroplast preparation. Spinach leaves gathered from a controlled climate facility were used to isolate chloroplasts according to the technique of Ort and Izawa [18]. Chlorophyll concentration was determined according to the method of Arnon [19].

Experimental. O_2 uptake was measured polarographically using a Clark-type oxygen electrode. The reaction mixture (2 ml) was contained in a 1 cm diameter water-jacketed vessel. The actinic light, provided by a 500 W tungsten-halogen lamp, was filtered by a $CuSO_4$ solution and a Corning CS 2-58 blocking filter.

Light-induced cytochrome absorbance changes were measured using a modified Aminco-Chance dual wavelength spectrophotometer. The signal from the photomultipler (EMI 9524A) together with a reference signal (480 Hz) were applied to a Princeton Applied Research HR-8 lock-in amplifier. The signal from the lock-in amplifier was recorded and stored in a digital signal averager (Tracor Northern NS-570). The half-rise time of the apparatus was 15 ms. The photomultiplier was

protected from stray light by a Balzers DT-G interference filter and a Corning CS 4-76 blocking filter. The intensity of the measuring beam was less than 5 ergs · cm^{-2} · s^{-1}. The actinic light intensity varied between 2.5 · 10^5 to 4.0 · 10^5 ergs · cm^{-2} · s^{-1} and was provided by a 150 W tungsten-halogen lamp, two glass heat filters and a Corning CS 2-58 blocking filter. The flash duration which varied between 0.6 and 1.6 s was determined by a Uniblitz model 26 electronic shutter and a model 310 drive unit. The far-red background light was filtered by a Balzers 732 nm interference filter with a 13 nm half-bandwidth. The intensity of the 732 nm light varied between 2 · 10^3 to 8 · 10^3 ergs · cm^{-2} · s^{-1}. The reference wavelength used was 570 nm. Before beginning measurements of cytochrome *b*-559 absorbance changes, the chloroplast sample was illuminated with several flashes in order to eliminate any changes unique to the first few flashes.

Ferricyanide reduction was measured in the instrument described above by observing the absorbance change at 420 nm with a reference wavelength of 450 nm. The photomultiplier was protected from stray light by Corning CS 5-57 and 4-96 blocking filters.

Light-induced changes in the ultraviolet region were measured using a DW2 spectrophotometer (American Instrument Co.) operated in a single beam mode. The photomultiplier signal was sent into a current amplifier and then recorded and stored in a Nicolet 1072 signal averager. The half-rise time of the apparatus was 4 ms. The photomultiplier (EMI 6255) was protected from stray light by a 260 nm Schott reflecting interference filter with a 20 nm half-bandwidth. The actinic light was provided by a lamp and filters identical to the system described above for the cytochrome measurements. The shutter and drive unit were both Uniblitz. The intensity of the far-red background light, filtered by a Balzers 720 nm interference filter with a 10 nm half-bandwidth, was approx. 3 · 10^3 ergs · cm^{-2} · s^{-1}.

All experiments were done at room temperature and chloroplasts were not used after standing at room temperature for longer than 10 min. All absorbance changes were measured using a cuvette with a 1 cm optical pathlength.

RESULTS

High potential cytochrome *b*-559 in chloroplasts is in the reduced state in the dark and light-induced absorbance changes are very small without pretreatment. In order to initially oxidize the cytochrome so that the kinetics of photoreduction could be studied, it was necessary to illuminate chloroplasts with relatively low intensity far-red actinic light in the presence of 1–2 μM PMS [16], or after preillumination with very high intensity actinic light [17]. Neither of these treatments had any observable effect upon the coupled or uncoupled electron transport rate of the chloroplasts. Photoreduction of chemically oxidized cytochrome *b*-559 could only be observed with very low concentrations of ferricyanide and the reduction rate was markedly slower than that measured after oxidation with low intensity far-red light (data not shown).

Typical kinetics of the light-induced absorbance change at 559 nm are shown in Fig. 1A. The spectrum of the absorbance change shows it to correspond to the reduced-oxidized difference spectrum of cytochrome *b*-559 (Fig. 2). The kinetics of the photoreduction, which appear to be first order, vary only slightly from one chloro-

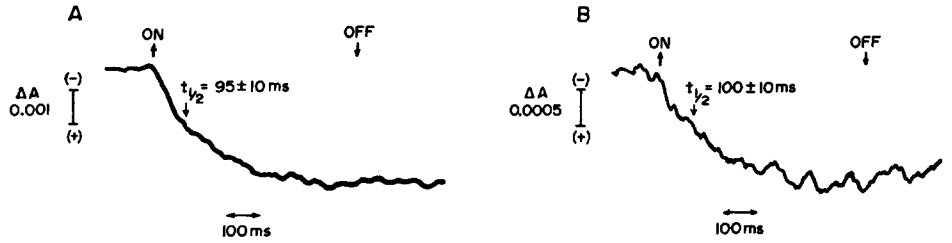


Fig. 1. (A) Kinetics of the absorbance change at 559 nm induced by illumination of spinach chloroplasts. The intensity of the actinic light was $3.5 \cdot 10^5$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1}. Lowering the intensity to $2.5 \cdot 10^5$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1} did not alter the extent or the rate of the absorption change which indicates that the intensity used was saturating. The sample was illuminated for 60–90 s prior to each flash by low intensity 732 nm light. The trace shown is the average of 20 runs, during which the sample was changed four times. The half-bandwidth of the measuring beam was 2.9 nm. The reaction mixture contained 0.2 M sucrose, 100 mM tricine/NaOH (pH 7.8), 2 mM $MgCl_2$, 100 μM methyl viologen, 2 μM PMS and 75 μg chlorophyll/ml. For further details, see Materials and Methods. (B) Kinetics of the absorbance change at 560 nm induced by illumination of spinach chloroplasts in the absence of PMS. The reaction mixture is the same as described in A except that it contained no PMS. The chloroplast sample was preilluminated for 30–60 s with white light ($2 \cdot 10^6$ ergs $\cdot$ cm$^2 \cdot$ s^{-1}) prior to beginning the measurements. The trace shown is the average of 15 runs during which the sample was changed three times. The intensity of the actinic light was $2.5 \cdot 10^5$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1}. The half-bandwidth of the measuring beam was 2.2 nm. Conditions were otherwise as described in A.

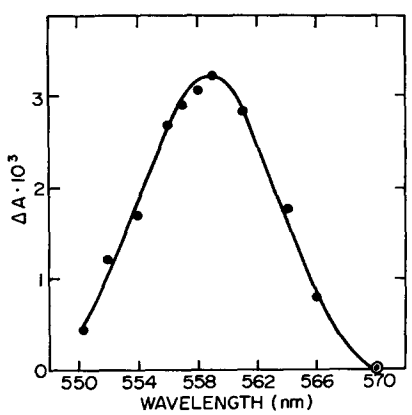


Fig. 2. Wavelength dependence of the light-induced absorbance change in chloroplasts shown in Fig. 1A. The measuring conditions were the same as described in Fig. 1A except the half-bandwidth of the measuring beam was 2.0 nm and the chlorophyll concentration was 100 μg/ml. The data were taken by alternately measuring the absorbance change at a given wavelength and at 559 nm. The ratio of the absorbance change at a given wavelength occurring between 125 and 500 ms after the onset of the flash to the similar change occurring at 559 nm is shown above. The ΔA indicated represents the average change in absorbance at 559 nm. The absorbance change occurring during the first 125 ms of the flash was not included in the calculation of the spectrum in order to eliminate the small, fast absorbance changes at 563 nm due to cytochrome b_6 reduction.

plast preparation to another with half-times falling between 90 and 110 ms. The photoreduction, but not the photooxidation, is completely blocked by 10 μM DCMU (data not shown). In both experiments PMS was added, which appeared to alter only the photooxidation of the cytochrome but not its photoreduction. As shown in Fig. 1B the half-time of the reduction in the absence of PMS is essentially the same as in its presence (Fig. 1A), the only difference being that in the absence of PMS the extent of the absorbance is one-half as large. The smaller extent of the reduction is a consequence of the fact that preillumination with high intensity light is not as efficient as the presence of PMS in stimulating the photooxidation, which means there is less cytochrome *b*-559 in the oxidized state at the onset of illumination. Although most of the experiments reported below were done in the presence of PMS in order to obtain appreciable turnover, comparison of Figs. 1A and 1B show that PMS does not effect the photoreduction of cytochrome *b*-559 and that the conclusions inferred from these data are valid for chloroplasts in the absence of PMS as well. In view of the labile nature of cytochrome *b*-559 we feel that it is important to point out that these experiments were done using chloroplasts capable of coupled electron transport. At the completion of the measurements shown in Fig. 1A the chloroplast preparation was assayed for oxygen evolution and exhibited rates (in μmol O_2/h per mg chlorophyll) of 56 and, in the presence of 5 μM gramicidin, 210.

The photoreduction shown in Fig. 1A represents an appreciable fraction of high potential cytochrome *b*-559. In the presence of PMS the cytochrome oxidized by far-red light is reducible by hydroquinone (data not shown). In the case of Fig. 1A, the reduction corresponds to approx. 70 % of the total high potential cytochrome *b*-559 present in our chloroplast preparation. Using a difference extinction coefficient of 15 mM^{-1} at 559 nm for a reference wavelength of 570 nm [1, 20], the stoichiometry of cytochrome *b*-559 photoreduced to chlorophyll is 1 : 375.

In Fig. 3 the kinetics, and in Fig. 4 the spectrum of the light-induced absorbance change attributed to plastoquinone reduction [21, 22] are shown. The half-time of plastoquinone photoreduction is indicated to be equal to or less than 10 ms because the half-rise time of the instrument was 4 ms. Our data show that the half-

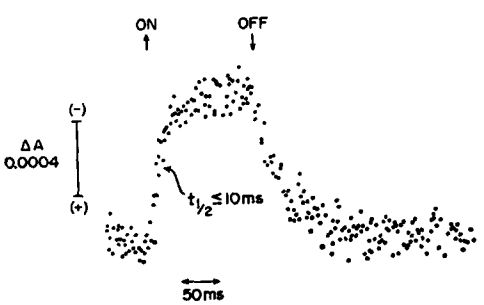
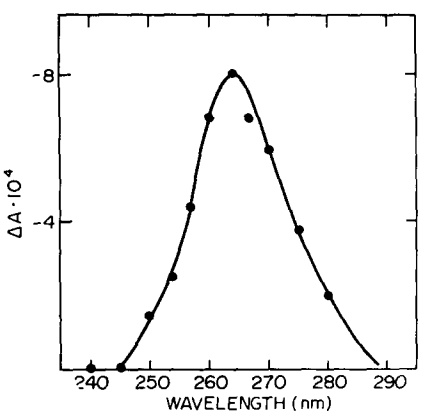


Fig. 3. Kinetics of the light-induced absorbance change at 264 nm. The sample was continuously illuminated by low intensity 720 nm light. The trace shown is the average of 1024 runs during which the sample was changed four times. The repetition rate of the flash was 2 Hz. The intensity of the actinic light was $3.0 \cdot 10^5$ ergs $\cdot$ $cm^{-2} \cdot s^{-1}$. The half-bandwidth of the measuring beam was 6 nm. The reaction mixture contained 0.2 M sucrose, 20 mM tricine/NaOH (pH 8.0), 5 mM $MgCl_2$, 20 mM KCl, 40 μM methylviologen and 20 μg chlorophyll/ml.



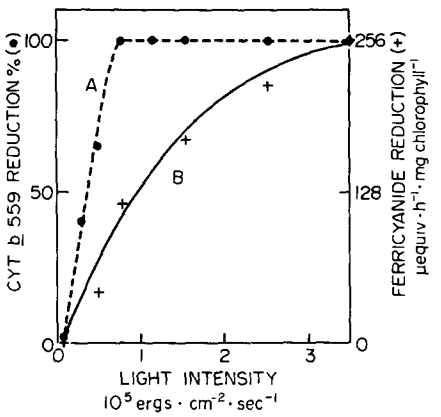


Fig. 4. Wavelength dependence of the light-induced absorbance change in chloroplasts shown in Fig. 3. The conditions were as described in Fig. 3. except the half-bandwidth was 2 nm. The spectrum was determined as described in Fig. 2 with the exception that the total absorbance change was calculated and the ratio was taken with respect to 264 nm.

Fig. 5. Comparison of the dependence of cytochrome *b*-559 reduction and ferricyanide reduction rate upon the actinic light intensity. (A) The percent reduction of cytochrome *b*-559 is defined as the ratio of the light-induced change of 561 nm at a given actinic light intensity to the change observed at maximum intensity. At $8 \cdot 10^4$ ergs $\cdot$ s^{-1} both the extent and the half-time of the reduction are unimpaired. At non-saturating intensities the half-time of the reduction increases. The reaction mixture contained 0.2 M sucrose, 100 mM tricine/NaOH (pH 7.8), 2 mM $MgCl_2$, 100 μM methyl viologen, 1 μM PMS, and 45 μg chlorophyll/ml. (B) Oxygen evolution was monitored by measuring the absorbance change at 420 nm corresponding to ferricyanide reduction during the first 0.5–1 s of a flash. The reaction mixture contained 0.2 M sucrose, 100 mM tricine/NaOH (pH 7.8), 2 mM $MgCl_2$, 0.25 mM ferricyanide, and 45 μg chlorophyll/ml. At the conclusion of the above measurements using actinic light of $3.5 \cdot 10^5$ ergs $\cdot$ cm^2 $\cdot$ s^{-1} the addition of 5μM gramicidin increased the rate of O_2 evolution by a factor of 2.0.

reduction time value lies between 6 and 10 ms and that the extent of the absorbance change corresponds to a turnover of three electrons per electron transport chain per flash.

A comparison of the dependence of cytochrome *b*-559 photoreduction and ferricyanide reduction on actinic light intensity shows half-saturation at intensities (in ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}) of approx. $3 \cdot 10^4$ and $1 \cdot 10^5$, respectively (Fig. 5). Complete light saturation of cytochrome *b*-559 photoreduction appears to occur at intensities (in ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}) of approx. $8 \cdot 10^4$ and ferricyanide reduction at $3 \cdot 10^5$–$5 \cdot 10^5$. This comparison was made using identical chlorophyll concentrations and illumination conditions. Cytochrome *b*-559 photoreduction by Photosystem II thus saturates at a light intensity approx. 1/3–1/6 of that at which ferricyanide reduction is saturated.

It has been shown that low concentrations of DBMIB inhibit electron transport on the system I side of the plastoquinone pool [23], and also inhibit photooxidation of cytochrome *b*-559 by Photosystem I [14]. In the presence of DBMIB (1 μM) the photoreduction of cytochrome *b*-559 occurs three to four times as fast ($t_{\frac{1}{2}}$ = 25–35 ms) as in its absence (Figs. 6 and 7). The amplitude of the absorbance change is smaller than that shown in Fig. 1 and is a consequence of the fact that DBMIB is a slow chemical reductant so that cytochrome *b*-559 was partially reduced during the

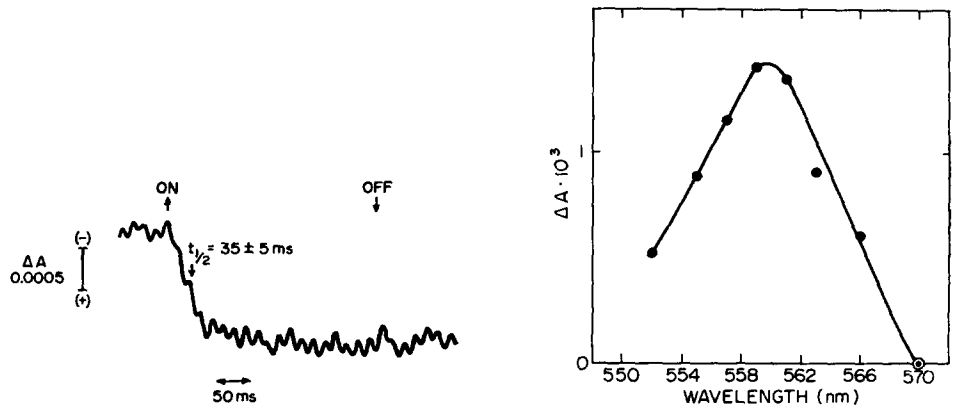


Fig. 6. Kinetics of the light-induced absorbance change at 559 nm in the presence of 1 μM DBMIB. The trace represents the average of 15 flashes. The sample was preilluminated with 732 nm light until cytochrome *b*-559 was oxidized. Then 1 μM DBMIB was added and a measurement was made within 15 s after the addition. The sample was changed after each flash. The intensity of the actinic light was $2.8 \cdot 10^5$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1}. The reaction contained 0.2 M sucrose, 30 mM tricine/NaOH (pH 7.8), 2 mM $MgCl_2$, 10 mM KCl, 100 μM methyl viologen, 1 μM PMS and 75 μg chlorophyll/ml.

Fig. 7. Wavelength dependence of the light-induced absorbance change in chloroplasts in the presence of 1 μM DBMIB as shown in Fig. 6. The conditions were as described in Fig. 6. The spectrum was determined as described in Fig. 2 with the exception that the total absorbance change was calculated.

time between addition of DBMIB and the onset of the flash.

The addition of uncouplers causes a further increase in the half-reduction time of cytochrome *b*-559. As shown in Fig. 8, in the presence of 5 μM gramicidin the half-reduction time was 156 ms compared to the control value of 93 ms. The extent of the absorbance change was unaffected by gramicidin. A similar effect was observed in the presence of NH_4Cl or nigericin (data not shown). The effect of gramicidin upon the rate of 0_2 evolution is shown in Table I using saturating light intensity and a light intensity comparable to that used in the experiment of Fig. 8. At the lower light intensity the rate of 0_2 evolution is not maximal and gramicidin causes a smaller increase in the rate.

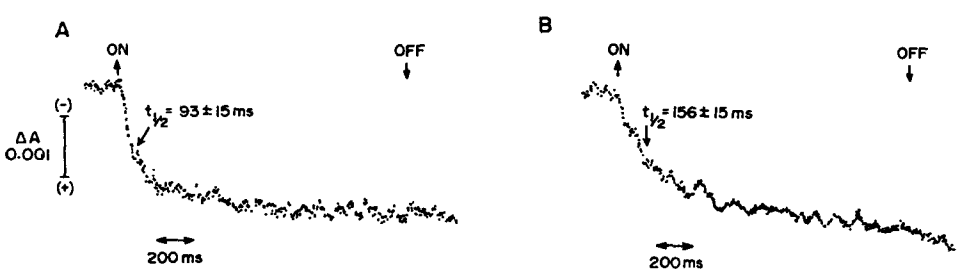


Fig. 8. The light-induced absorption change at 559 nm in the absence (A) and in the presence (B) of 5 μM gramicidin. The half-bandwidth of the measuring beam was 2 nm. Each trace is the average of 15 flashes during which the sample was changed three times. The intensity of the actinic light was $4 \cdot 10^5$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1} and for trace B was not saturating. The reaction medium was as described in Fig. 6.

TABLE I

THE EFFECT OF GRAMICIDIN ON OXYGEN EVOLUTION UNDER CONDITIONS OF SATURATING AND NON-SATURATING LIGHT

The intensity under "high light" was greater than $2 \cdot 10^6$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1} and was saturating. The intensity under "low light" was approx. $4 \cdot 10^5$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1}. The reaction medium was as described in Fig. 6 with the addition of 1 mM NaN_3. The addition of 1 μM PMS did not alter the results shown below.

Conditions	O_2 evolution in μmd $\cdot$ h$^{-1} \cdot$ mg^{-1} chlorophyll	
	High light	Low light
Control	49	36
+5 μM gramicidin	139	54

In the presence of ADP plus P_i the rate of cytochrome *b*-559 photoreduction is slightly slower than the control. However, the kinetics are more complex and appear to be biphasic. In the presence of ATP, although the photoreduction rate appears to be faster, it is not significantly different from the control (data not shown).

DISCUSSION

In the generally accepted model for photosynthetic electron transport, electrons are considered to be transferred from Q, the primary acceptor of Photosystem II, to plastoquinone, and then, via cytochrome *f* and plastocyanin, to *P*-700, the reaction center chlorophyll of Photosystem I. The rate-limiting step has been shown to be between plastoquinone and cytochrome *f* and is typically 20 ms [21]. In addition to the above components there has appeared in the literature the suggestion that a redox component functions between Q and plastoquinone, the identity of which is not agreed upon [3, 24–26]. Any such component, in order to be compatible with the fast reduction of plastoquinone, must meet certain kinetic requirements. Plastoquinone, in single turnover flash experiments, receives an electron from Photosystem II in less than 1 ms [21]. However, using longer flash times it takes approx. 10 ms for the plastoquinone pool to become reduced. Therefore, an intermediate carrier, between Q and plastoquinone, must be capable of transferring electrons at a rate comparable to the rate of plastoquinone reduction. Although the onset of the reduction of an intermediate may be delayed due to the time it takes to reduce the plastoquinone pool, the reduction should then proceed very rapidly and must certainly be faster than the rate-limiting step of 20 ms. In the experiments described here the half-time for the photoreduction of cytochrome *b*-559 is typically 100 ms; whereas the half-time for the photoreduction of plastoquinone is 10 ms or less. These data show that cytochrome *b*-559 does not meet the requirements for an intermediate component as described above and therefore imply that cytochrome *b*-559 does not function as an obligatory redox component in the electron transport chain between Q and plastoquinone. This is also implied by the saturation of cytochrome *b*-559 photoreduction before saturation of non-cyclic electron transport.

It might be argued that the relatively slow photoreduction of cytochrome

b-559 is a consequence of dark initial conditions, and that the photoreduction may proceed more rapidly under steady-state conditions. Relevant to this argument is recent work which shows that steady-state rates of phosphorylation are established within 50 ms after the onset of illumination under conditions very similar to those employed here [27]. The half-reduction time of 100 ms and the smooth time course for photoreduction implies that the relatively slow rate of cytochrome *b*-559 photoreduction also applies to the steady-state level of energy coupling which exists after 50 ms of illumination.

Based on a comparison of the half-time of cytochrome *b*-559 reduction and a maximum half-time for reduction of the plastoquinone pool, the present experiments imply that cytochrome *b*-559 is an acceptor for no more than one of every 10 electrons accepted by the PQ pool. The effect of uncouplers and ADP+P_i in slowing the reduction of cytochrome *b*-559 implies that cytochrome *b*-559 interacts with plastoquinone to a degree sufficient for electron flow through coupling site I [28] to affect the steady-state redox level of the cytochrome. These data lead to the general conclusion that cytochrome *b*-559 in a branched pathway is reduced by either Q, a component between Q and plastoquinone, or plastoquinone.

It has previously been argued by others that cytochrome *b*-559 is not a component in the main chain joining the two photosystems [4, 29–31]. The presence of this cytochrome could not be seen in difference spectra of whole cells of *Porphyridium aerugineum* and *Chlorella vulgaris* alternately illuminated with non-saturating red and far-red actinic light [29]. It is a general observation in chloroplasts, as well as whole algal cells, that the amplitude of photooxidation of the cytochrome is generally very small in the absence of special additions or pretreatments. In seeking the function of this cytochrome, we have previously considered the possibility that the cytochrome does function in the main chain, but that the conditions of most spectrophotometric measurements were not optimum for observation of the function, which might require a transient negatively directed shift in the midpoint potential of the cytochrome [3]. We have obtained evidence for an irreversible shift in the midpoint potential of cytochrome b_6 after illumination in the presence of uncouplers [32], and for a relatively small reversible shift in potential of cytochrome *b*-559 in the dark when the pH is lowered to 5.0 [33]. We have found several treatments which cause an increase in the amplitude of oxidation by far-red light, and have inferred that the oxidation involved a lower potential form of the cytochrome because it was inhibited by DBMIB. The ability of DCMU at low concentrations to inhibit an acid-induced decrease in midpoint potential of cytochrome *b*-559 implied a direct interaction of DCMU with cytochrome *b*-559 [34]. Since DCMU is an efficient inhibitor of main chain electron transport, this again suggests that the cytochrome might function in the main chain. However, it has not been possible to demonstrate a rapid oxidation of cytochrome *b*-559 by far-red light, at least partly because the far-red light intensities which are optimum for studying the cytochrome *b*-559 oxidation are too small to saturate Photosystem I. Since for these reasons it was not possible to study the kinetics of the far-red oxidation, the purpose of the present study was to study the kinetic sufficiency of reduction of cytochrome *b*-559 by red light. As discussed above, the results of these experiments do not fit with the hypothesis that cytochrome *b*-559 is an obligatory redox component in the main chain.

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