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Slow electrogenic phase and intersystem electron transfer in algae

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Chlorella sorokiniana **briefly treated with 0.3 mM benzoquinone which oxidized the plastoquinone pool were submitted to a series of laser flashes. After each flash, a fast increase of the membrane potential (phase a) is followed by a slow increasing phase (phase b; $t_{1/2}$, 70 ms), the amplitude of which oscillates with a period 2. This amplitude is close to zero after the first flash and exceeds one electron per electron-transfer chain after the second flash. The amount of electrons transferred to Photosystem I donors (cytochrome *f*, plastocyanin and P-700) oscillates with a period 2 in synchrony with phase b. A few seconds after even flashes, the absorption level in the visible range is close to the one measured on dark-adapted sample, which means that no electron storage occurs in any of the carriers involved in the intersystem electron transfer. After even flashes, a large reduction of cytochrome *b* is observed which precedes the reduction of Photosystem I donors. Living algae, i.e., untreated with benzoquinone, were incubated under anaerobic conditions which induced the reduction of more than one molecule of cytochrome *b* per cytochrome *b/f* complex. A large oxidation of cytochrome *b* is observed after the first flash, while a second flash induced a transitory reduction followed by a slow reoxidation. Each of the two flashes generates a large phase b ($t_{1/2}$, 5 ms) with similar amplitude and kinetics. After the second flash, the reduction of cytochrome *b* is almost synchronous with the oxidation of cytochrome *f*, in apparent contradiction with the concept of concerted reduction of cytochrome *f* and cytochrome *b*. A model is proposed in which the electrogenic phase is associated with the protonation of the reduced Fe-S Rieske protein, which implies the transfer of one proton across the membrane. This electrogenic phase would be the time-limiting process and responsible for the delay in the reduction of Photosystem I donors. Such a proton-pumping mechanism does not exclude that a Q cycle could operate under other experimental conditions.**

Introduction

Electron transfer between the two photosystems involves two diffusible carriers, plastoquinone (PQ) and plastocyanin, and a transmembrane complex which includes at least one cytochrome *f* (Cyt *f*), two b_6 cytochromes (Cyt *b*) and the Fe-S Rieske protein. Electrons are transferred from PS II acceptors to Cyt *b/f* complex by plastoquinol which diffuses in the lipid phase of the membrane. Electrons are transferred from Cyt *b/f* complex to PS I via plastocyanin, which diffuses in the internal aqueous phase of the thylakoid. A slow flash-induced increase of the membrane potential (phase *b*) has been first observed in living algae [1–3] and then in isolated chloroplasts [4]. For each PS I reaction, one extra-electron is trans-

Abbreviations: PS, Photosystem; PQ, plastoquinone; cytochrome *b* or Cyt *b*, cytochrome *b*-563; Cyt *f*, cytochrome *f*; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; Q_B, secondary acceptor of Photosystem II.

ferred by this slow electrogenic process [2]. Velthuys [5] and Bouges-Bocquet [6] later reported that it was also involved in the intersystem electron transfer, as the amplitude of phase b oscillates with a period 2 along a flash series. Fowler and Kok [7] and Velthuys [4] showed that the transfer of one extra proton was associated with the transfer of one electron from PS II acceptors to PS I donors.

According to Mitchell [8], the Cyt *b/f* complex includes an electrogenic loop (Q-cycle) which allows the oxidation of plastoquinol at a site 'Z' close to the inner face of the membrane and the reduction of a plastoquinone at a site 'C' close to the outer face of the membrane (Scheme I). The analysis of the structure of the Cyt *b/f* complex has shown that the two Cyt *b* are located close to the inner and the outer faces of the membrane, respectively [9,10]. If we accept the Q cycle model, it is then likely that the electrogenic process corresponds mainly to the transfer of one electron between the two Cyt *b*. The absence of an electrogenic phase associated with the oxidation of Cyt *f* by PS I shows that Cyt *f* is located on the same side of the membrane as P-700, i.e., on the inner face.

Other models, as those of Wikström and Krab [11] or Papa et al. [12] have been proposed to describe the process of electron transfer in the Cyt *b/f* complex. These models could include true proton-pumping devices coupled with some of the electron-transfer reactions.

A common feature to all the interpretations is the process of a concerted reduction of Cyt *b* together with PS I secondary donors (Fe-S or Cyt *f*?) by plastoquinol at site Z. We recently studied the kinetics of this phenomenon in isolated chloroplasts under oxidizing conditions [13,14]. Whereas in the recent literature the Q cycle or its modified version [15] is the most popular model, the experiment of Girvin and Cramer [16], performed under highly reducing conditions represents a serious challenge to this class of models: these authors observed an unmodified slow electrogenic phase under conditions in which Cyt *b* is totally reduced prior to a flash illumination.

In this paper, we investigate the slow electrogenic process (phase b) and the associated electron-transfer reactions in different strains of *Chlorella sorokiniana* kindly provided by Bennoun.

Material and Methods

The experiments were performed on different strains of *Chlorella sorokiniana*. The diameter of this algae is smaller than 3 μm, which makes this material specially well-adapted for spectrophotometric studies. The five strains we used are the wild-type, mutant S8 which lacks PS II, mutant S11 which lacks PS I, mutant S30 with a normal electron-transfer chain but a reduced antenna size and mutant S52 which lacks PS II and a large part of the antenna. All these mutants have been characterized from biochemical, structural [17] and biophysical [18] studies. Algae were used either untreated, or treated with benzoquinone as follows: algae were incubated for 8 min in the presence of 3 mM benzoquinone. Algae were centrifugated during this period at the end of which they were resuspended in 0.05 M phosphate buffer (pH 6.5)/0.1 M sucrose. Quinone treatment is known to make the cell and the chloroplast membranes permeable to ions [19,20]. The low concentration of quinone we used does not induce the fixation effects reported in Ref. 19. In all cases (treated and untreated algae), 10% ficoll was added in order to minimize the settling and the light scattering of this material.

Spectrophotometric measurements were performed with an apparatus similar to that described in Ref. 21 and improved according to Ref. 13. Actinic excitation is provided by a dye laser flash Candela SLL150. The emission peaks at 692 nm (oxazine) and the total duration of the flash (approx. 700 ns) is short enough not to induce double photoreactions.

The variations of the membrane potential are followed by the electrochromic shift of membrane pigments. The field-indicating absorption change was measured by the difference $\Delta I/I$(515 nm) − $\Delta I/I$(474 nm).

We measured the relative number of PS I and PS II centers on wild type and S30, by comparing the fast flash-induced field-indicating changes (phase a) when both photosystems are active and when PS II is blocked by hydroxylamine and DCMU: PS I and PS II are present at the same concentration, within variations of less than 10%.

The measurements of the redox changes of electron carriers were preferentially performed in

mutants with a small antenna size. It actually permits to increase the sensitivity by using higher concentrations of material. On the other hand, the perturbations due to the electrochromic shift are lowered and may be neglected for wavelengths longer than 545 nm.

The redox changes of Cyt *b*, Cyt *f* and plastocyanin were measured as described in Ref. 13. P-700 for the wild-type and mutant S52 was measured at 487 and 495 nm, respectively, wavelengths which correspond to the isosbestic point in the spectrum of the electrochromic effect. A correction was applied to take into account the small contribution of plastocyanin at these wavelengths, according to the spectrum of plastocyanin given in Ref. 22.

For Cyt *f*, the extinction coefficient $\varepsilon_{554-565\,nm}$ is 23 $mM^{-1} \cdot cm^{-1}$ [23]; in the conditions of our deconvolution procedure, the corresponding value of ε is 20 $mM^{-1} \cdot cm^{-1}$. For Cyt *b*, Stuart and Wasserman [24] give $\varepsilon_{563-600\,nm}$ equal to 17 $mM^{-1} \cdot cm^{-1}$, while Nelson and Neumann [23] give $\varepsilon_{563-575\,nm}$ equal to 24.4 $mM^{-1} \cdot cm^{-1}$; the corresponding values computed for our deconvolution procedure are 12.8 and 18.8 $mM^{-1} \cdot cm^{-1}$, respectively.

The signal which corresponds to a full oxidation of Cyt *f* was measured by submitting quinone-treated algae to a continuous illumination. We checked that in dark-adapted quinone-treated material, Cyt *f* was fully reduced.

The experiments were performed at room temperature unless otherwise indicated.

Results

Quinone-treated algae

Effect of quinone treatment. Fluorescence induction curves were measured on quinone-treated wild type and on S11 strain (Table I). As in isolated chloroplasts, the fluorescence increase is biphasic and the slow phase (difference between columns A and B, Table I) is associated with the reduction of the plastoquinone pool. For the wild type in the absence of electron acceptor, the maximum fluorescence yield is lower than in the presence of DCMU, which indicates that the process which allows PS I electron-acceptor reoxidation has not been totally deactivated. Addition of ferricyanide decreases the steady-state fluorescence yield, showing that after quinone treatment this electron acceptor has a free access to some of the PS I acceptors. In agreement with Refs. 19 and 20, we concluded that quinone treatment made the cell and the chloroplast membranes permeable to ions. Surprisingly, methyl viologen at a concentration as high as 0.4 mM does not accept electrons from PS I donors. One thus must conclude that methyl viologen has no access to the low-potential PS I acceptors (less than −400 mV). In mutant S11 which lacks PS I, the maximum fluorescence yield is high and only slightly decreased by the addition of ferricyanide.

TABLE I

VARIABLE FLUORESCENCE YIELD F_V/F_O MEASURED ON QUINONE-TREATED ALGAE

F_O, fluorescence yield measured on dark-adapted sample; F_V, fluorescence yield minus F_O. Intensity of the continuous beam: approx. 15 photons per s per PS II center. Wild type: (A), amplitude of the fast phase after 0.2 s continuous illumination. (B), maximum fluorescence yield reached after 5 s continuous illumination. S11: (C), same as B. Mutant S11 strain lacks PS I.

	Wild type		S11
	A	B	C
No addition	0.81	1.55	2.66
0.2 mM ferricyanide	0.64	0.77	2.31
0.2 mM methyl viologen	0.74	1.42	
20 μM DCMU		2.60	

A second effect of quinone treatment is the inhibition of the ATPase activity, as shown by Diner and Joliot [25]. Under these conditions, the decay of the light-induced field indicating change is particularly slow ($t_{1/2}$, 2–4 s); this slow decay also shows that the permeability of the thylakoid membrane has not been increased by the quinone treatment. A similar conclusion may be reached from the fact that the PS I donors (Cyt *f*, plastocyanin, P-700) are not oxidized by a long exposure (more than 4 h) to 0.2 mM ferricyanide. Unexpectedly, addition of a mediator such as DAD does not facilitate the oxidation of PS I donors by ferricyanide.

A third effect of quinone treatment is to block totally the respiratory pathway previously characterized by Bennoun [26], as well for the input of electrons into the plastoquinone pool as for their

output via the oxidase. Consequently, the plastoquinone pool is fully oxidized. The absence of plastoquinol on dark-adapted material simplifies the study of the intersystem electron transfer.

Generation of the membrane potential by a flash series. Fig. 1 shows the effect of one or two flashes given to quinone-treated algae. In the absence of DCMU, phase a ($t_{1/2} < 1$ μs) is followed by the slow increasing phase b, the amplitude of which is larger after the second than after the first flash. This slow phase is slower ($t_{1/2}$ 70 ms) than that observed in living material or in chloroplasts under reducing conditions ($t_{1/2} \approx 2$–4 ms), i.e., in the presence of preexisting plastoquinol. In the presence of DCMU, phase b totally disappears. One can conclude that (a) no plastoquinol is present on dark-adapted material and (b) in the absence of DCMU plastoquinol is formed by PS II, likely via the two electron gate mechanism [27].

The measurement of both the amplitude and the kinetics of phase b is complicated by the superposition of the membrane-potential decay. We used the following assumptions to deconvoluate phase b: (1) Phase b does not last for times longer than 500 ms: (2) if V is the value of the membrane potential (relative units), we established after each flash the function $dV/dt = f(V)$ during the decay of the membrane potential in the 500 ms to 2.5 s time-range.

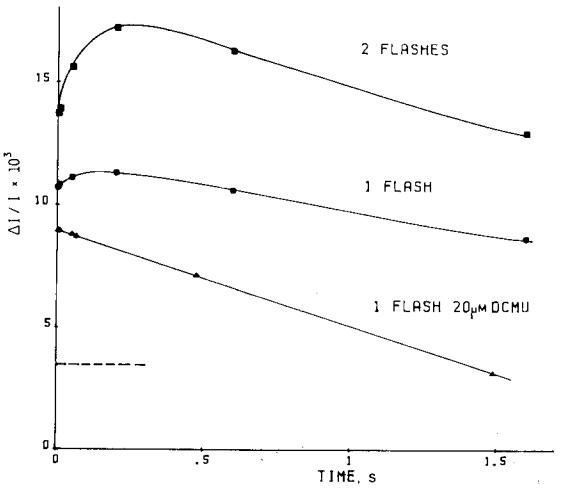


Fig. 1. Time-course of the field-indicating absorption change $\Delta I/I$(515 nm) minus $\Delta I/I$(474 nm) after one or two flashes 7 s apart. Quinone-treated algae. Dashed line, absorption level 50 ms before the second flash.

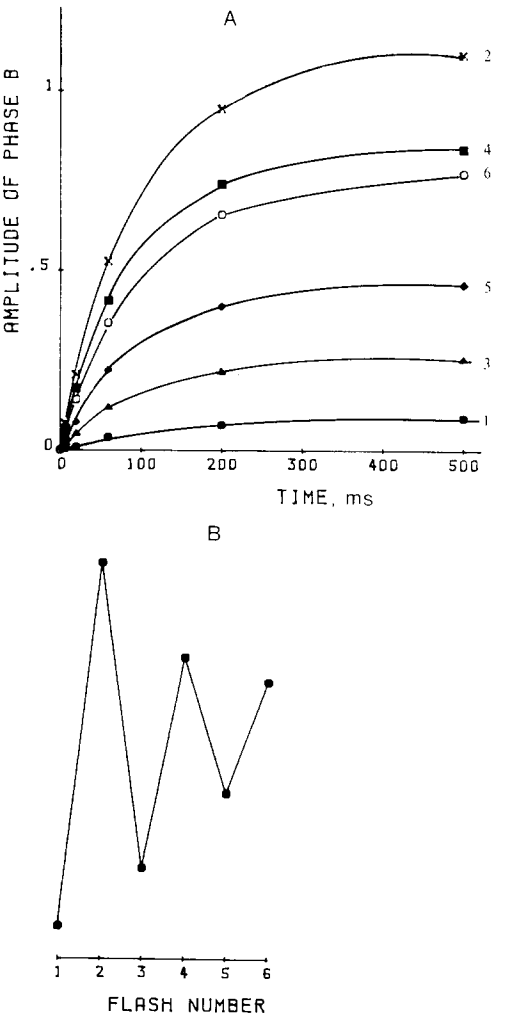


Fig. 2. (A) Time-course and (B) amplitude of phase b after 1–6 flashes (Fig. 2A, curves 1–6) 6 s apart. Quinone-treated algae were incubated for more than 30 min in the presence of 0.2 mM ferricyanide. See text for the deconvolution procedure. Phase b is expressed in terms of the number of charges transferred across the membrane per electron-transfer chain: its amplitude has been divided by twice the amplitude of phase a induced by the first flash, which corresponds to the transfer across the membrane of two electrons per electron-transfer chain.

Fig. 2 shows the kinetics and the amplitude of phase b, respectively, after 1–6 flashes in the presence of 0.2 mM ferricyanide. (1) In spite of the long time interval between flashes (6 s), period 2 oscillations with moderate damping are observed. These oscillations are more pronounced than those

previously reported in Refs. 5 and 6. (2) As expected, the amplitude after the first flash is decreased by addition of ferricyanide (oxidation of Q_B^-). A subsequent addition of DAD decreases the amplitude of phase b to zero (data not shown). (3) The half-time of phase b ($t_{1/2} \approx 70$ ms) is roughly independent of its amplitude, and its kinetics is close to an exponential function, which is characteristic of a first-order process. (4) The amplitude of phase b was normalized to the amplitude of phase a measured after the first flash. On dark-adapted material, phase a corresponds to the transfer of two electrons across the membrane (one electron for each photosystem); then, in the 0–500 ms time range after the second flash, phase b corresponds to the transfer across the membrane of 1.1 electrons per electron-transfer chain. (5) A comparison of the decay of the membrane potential after the first flash and the subsequent flashes suggests that phase b includes a non negligible 'tail' in the 500 ms–2.5 s range, which is eliminated by our deconvolution procedure. We estimate the amount of electrons transferred after the second flash in this time-range included between 0.15 and 0.3 electrons per electron-transfer chain.

In Fig. 3 is plotted the 515 nm absorption change measured after a group of two flashes minus 1 flash. This difference eliminates a small variation of the membrane potential associated with the reoxidation of the primary PS II quinone acceptor which occurs in the 0–3 ms time-range. No delay longer than 1 ms is observed in the course of phase b.

In the preceding experiments, the only available plastoquinol was formed by PS II. In the experiment of Fig. 4, the flash-induced formation of the membrane potential was measured in the presence of sodium dithionite, which slowly reduced the plastoquinone pool. For short times of incubation (curve 1), the amplitude of phase b is small (less than one charge transferred per photocenter) which means probably that less than one molecule of plastoquinol per photocenter is in its reduced state. Nevertheless, the half-time of phase b ($t_{1/2} \approx 10$ ms) is considerably shorter than those in the experiments of Figs. 1 and 2. For times of incubation longer than 20 min, the amplitude of phase b is about one electron per electron-transfer chain and its half-time decreases to a limit value of 2.5 ms.

Flash-induced redox changes of PS I donors. In the experiment of Fig. 5, the algae were submitted to a series of four flashes 7 s apart. The spectral changes associated with the variations of the redox states of plastocyanin, Cyt *f* and P-700 were mea-

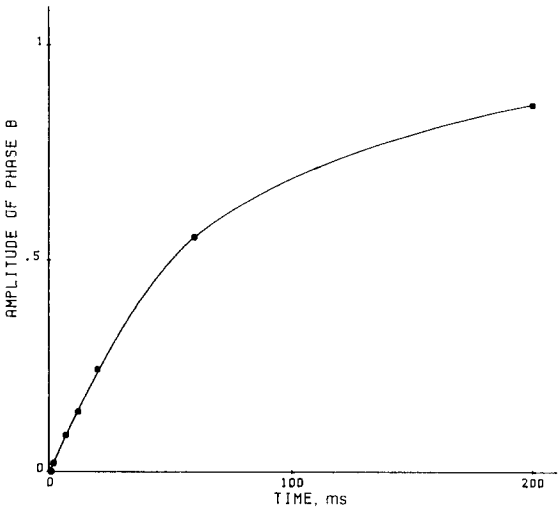


Fig. 3. Slow electrogenic phase measured after a group of two flashes minus one flash. Same conditions as in Fig. 2.

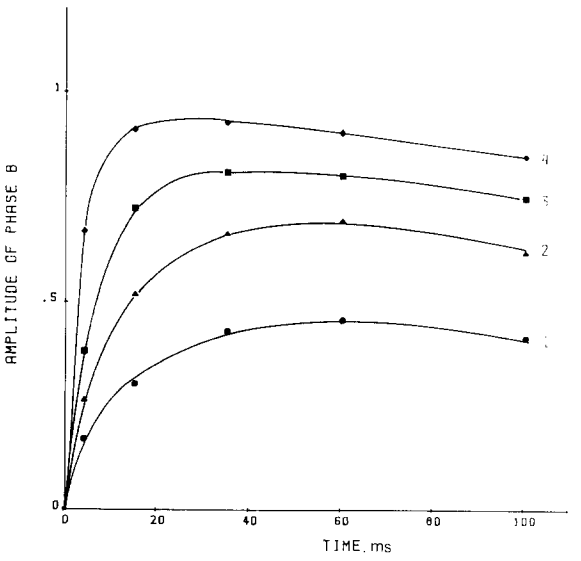


Fig. 4. Time-course of phase b after a flash in the presence of 0.4 mM sodium dithionite. Time of incubation: curve 1, 3 min; curve 2, 5 min; curve 3, 7 min; curve 4, 19 min. Amplitude of phase b: same units as in Fig. 2; phase a was measured before the addition of dithionite.

sured after each flash of the series (Fig. 5A–C). In order to minimize the contribution of the field-indicating spectral change, this experiment was performed in the presence of 0.2 mM dicyclohexyl-18-*crown*-6 which accelerates the decay of the membrane potential in algae (Diner, B.A., personal communication). The comparison of the experiments performed with and without dicyclohexyl-18-*crown*-6 shows that the kinetics of electron-transfer reactions are rather independent of the value of the membrane potential (data not shown). On Fig. 5D, the amplitude of phase b measured in the absence of dicyclohexyl-18-*crown*-6 has been plotted in the 0–500 ms range. The following conclusions may be drawn from these experiments: (1) The amount of electrons transferred to PS I oscillates with a period 2 in synchrony with the oscillations of phase b. (2) Most of the electrons are transferred during the first 500 ms following each flash: nevertheless, in the 500 ms–5 s range, a slow electron transfer is observed. This slow injection of electrons is likely associated with the 'tail' in phase b mentioned above. (3) 5 s after the even flashes, the absorption reaches a value close to the one measured on dark-adapted sample (base line). This shows that the number of positive charges formed on PS I donors is almost equal to the number of electrons transferred from PS II to PS I.

Fig. 6 shows the kinetics of the redox changes of P-700, plastocyanin, Cyt *f* and Cyt *b* induced by one or two flashes 7 s apart given to a mutant (S30) with a low chlorophyll content in the presence of dicyclohexyl-18-*crown*-6. After the first flash (Fig. 6A), a large oxidation of PS I donors is observed. A second flash (Fig. 6B) is required to

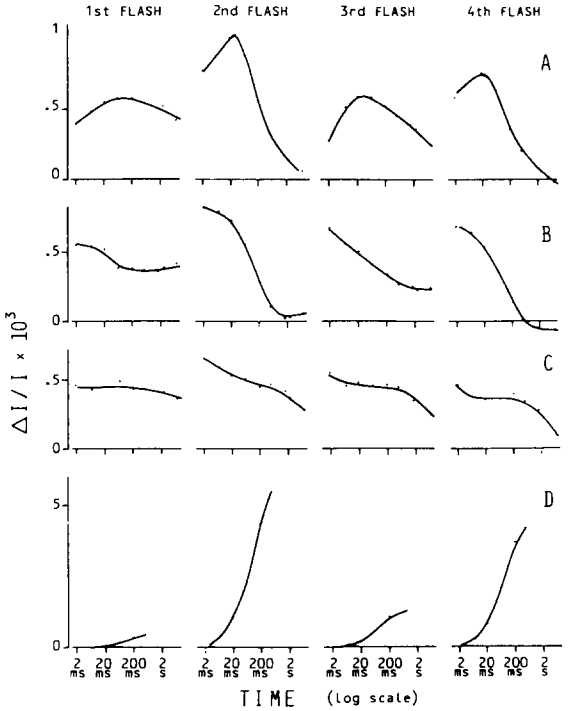


Fig. 5. Time-courses of the changes of the redox states of (A) plastocyanin, (B) Cyt *f*, (C) P-700 and (D) of the slow electrogenic phase upon illumination by 1–4 flashes 7 s apart. Quinone-treated wild-type algae (same batch as in the experiment of Fig. 2). 0.2 mM ferricyanide. Logarithmic time scale. In experiments of Fig. 5A–C, 0.2 mM dicyclohexyl-18-*crown*-6 was added. For each Photosystem I donor, the absorption change is plotted so that increasing values corresponds to increasing oxidized state.

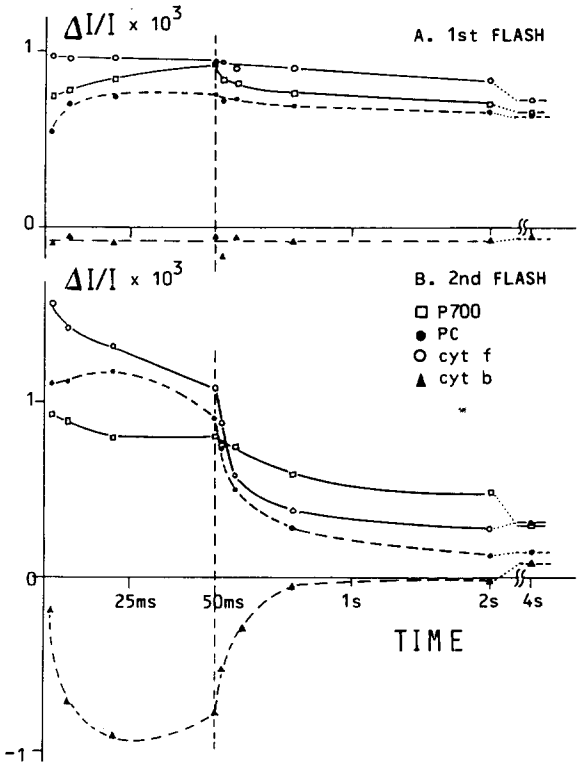


Fig. 6. Time-courses of the changes of the redox states of P-700, plastocyanin (PC), Cyt *f* and Cyt *b* induced by 1 (A) or 2 (B) flashes 7 s apart. Quinone-treated mutant S30. 0.2 mM ferricyanide; 0.2 mM dicyclohexyl-18-*crown*-6.

induce a reduction of PS I donors and a transient reduction of Cyt *b* (approx. 0.7 molecules per Cyt *b/f* complex). From 2 to 7 ms, there is no significant reduction of P-700 and plastocyanin: the amount of electrons transferred to Cyt *b* is 3.5–5.5 times higher than that transferred to Cyt *f* depending upon the value chosen for the Cyt *b* extinction coefficient (see Materials and Methods). Phase b (data not shown) essentially correlates with the reduction of Cyt *f* and the oxidation of Cyt *b*. The reduction of Cyt *f* is roughly synchronous with the reduction of the other PS I donors, which shows that the apparent equilibrium constants between these carriers are close to 1.

When performed in the absence of dicyclohexyl-18-*crown*-6, this experiment leads to similar results except the presence of a small peak around 560 nm, the origin of which is unknown.

The same experiment has been performed at low temperature (5°C). 3 s after the second flash, Cyt *f* has been fully rereduced while a large fraction (more than 30%) of P-700 and plastocyanin stays in the oxidized form for times longer than 6 s (data not shown). This suggests that in this particular case, either the equilibrium constant for the electron transfer between Cyt *f* and P-700 is lower than 1 or the transfer of electrons between these two carriers is transitorily interrupted.

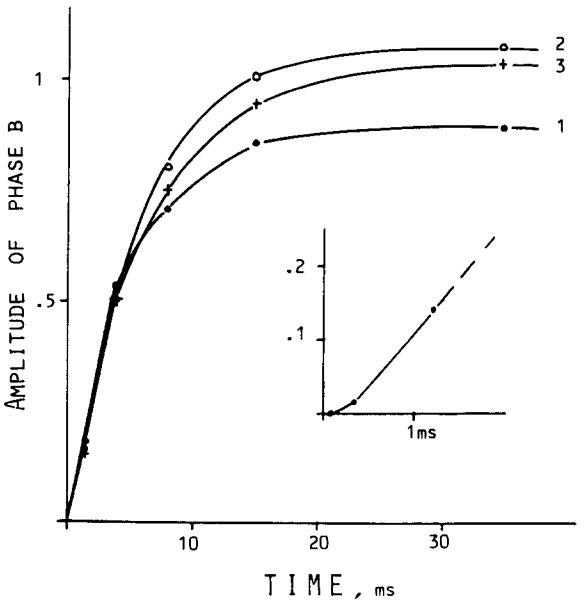


Fig. 7. Time-course of phase b measured after flash excitation on mutant S8. The amplitude of phase b is divided by the amplitude of phase a which, on mutant S8, corresponds to the transfer across the membrane of one electron per PS I center. Temperature 2°C. Curve 1 Aerobic conditions. From fluorescence measurements, we estimated that 10–20% of the plastoquinone pool is reduced. Curve 2 Anaerobic conditions. The sample is equilibrated with nitrogen and then maintained in the measuring cuvette in the presence of 20 mM glucose and 2 mg/ml glucose oxidase 30 min before the flash. Curve 3, same as 2, but after a second flash 5 s apart.

Untreated algae

Phase b. One must first recall that on dark-adapted living algae, a fraction of the plastoquinone pool stays in its reduced form due to the permanent injection of electrons by the respiratory pathway [26]. Fluorescence kinetics measurements showed that plastoquinol represents 10–50% of the total pool at room temperature and 5–20% at 2°C. Fig. 7 shows the time-course of phase b measured after one saturating flash given under aerobic conditions or after one and two flashes under anaerobic conditions in mutant S8 which lacks PS I: the half-time ($t_{1/2} \approx 4$ ms) of phase b is rather independent of the state of oxidation of the pool, as already stated by Bouges-Bocquet [28]. It also must be noted that under anaerobic conditions, phase b is very similar after the first or the second flash, both in amplitude and kinetics. A lag (inset Fig. 7), the duration of which is shorter than 300 μs, is probably linked to the oxidation time of the Fe-S protein.

If for a given batch the characteristics of phase b are rather independent of the experimental conditions, a large variability is observed from one algal culture to the other. The ratio of the amplitudes phase b/phase a is always maximum under anaerobic conditions and, in mutant S8, can reach values as high as 1.3 after a saturating flash and 1.45 after a nonsaturating flash. These values are significantly higher in mutant S52, but we cannot exclude that in this case the contribution of electrochromic probes with a quadratic response becomes significant in this mutant devoided of a large part of the chlorophyll antenna.

Flash-induced spectral changes in the green region of the spectrum. The experiments of Figs. 8 and 9 were performed at 2°C in anaerobiosis on a double-mutant strain (S52) which lacks PS II and a large part of the chlorophyll antenna. After a 1st

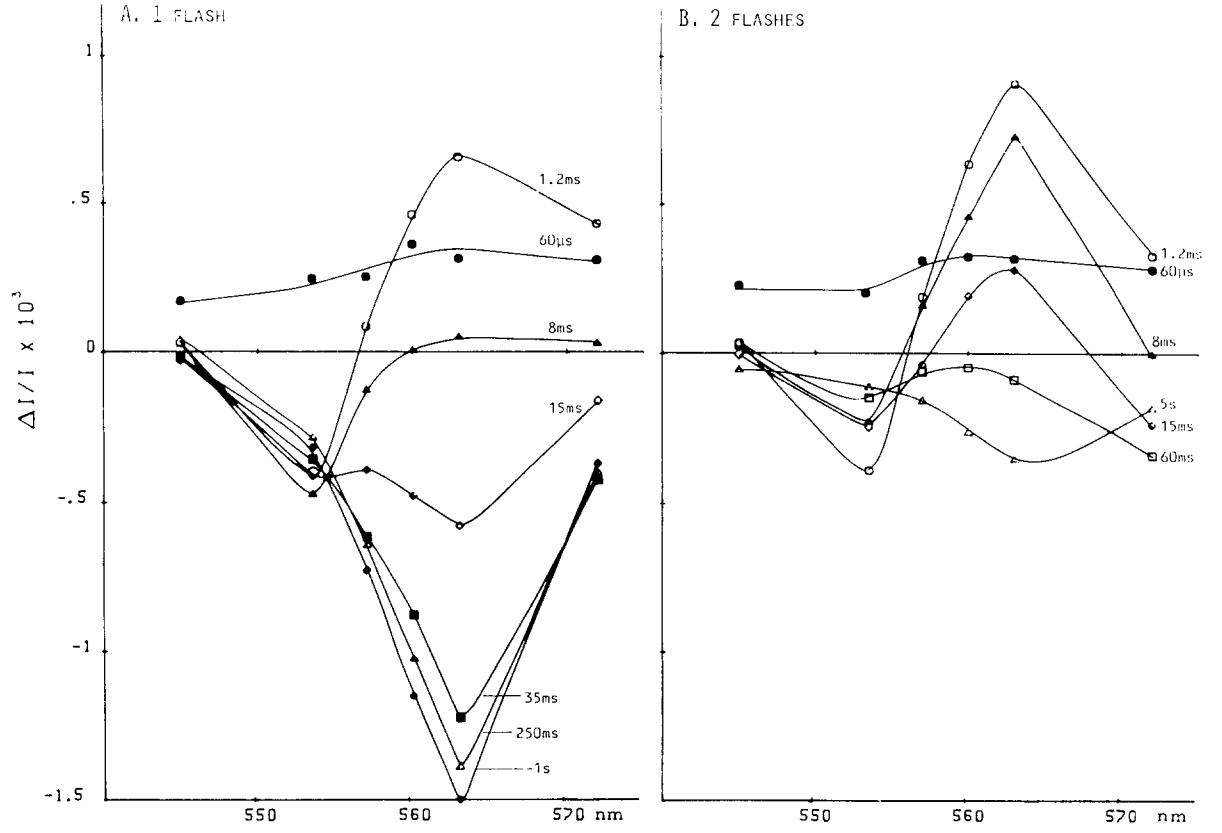
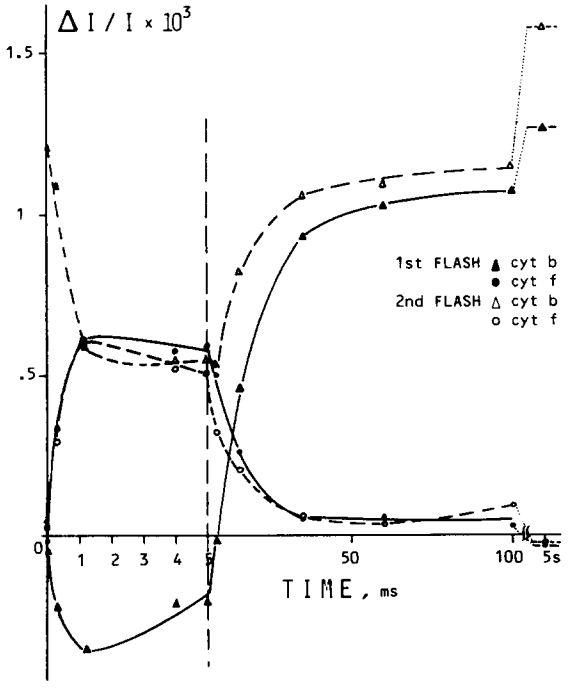


Fig. 8. Absorption changes induced by one flash (A) or two flashes (B) 5 s apart given under anaerobic conditions as described in Fig. 7. Mutant S52. Temperature, 2°C. In (B), the base line was taken 100 ms before the second flash.



flash, a small and fast phase of reduction of Cyt *b* is followed by a large oxidation. A maximum amount of 0.7 and 0.9 molecules of Cyt *b* per Cyt *b/f* complex is oxidized in the absence or in the presence of dicyclohexyl-18-*crown*-6, respectively. After the first flash, the rereduction of Cyt *b* is a slow process ($t_{1/2} \approx 1$ min), which explains why in these experiments we had to use long time-intervals (4 min) between two successive groups of two flashes.

A second flash given 5 s after the first one induces a reduction of Cyt *b* which largely pre-

Fig. 9. Time-course of the variation of the redox state of Cyt *b* and Cyt *f* after one or two flashes 5 s apart under anaerobic conditions. Same experiment as in Fig. 8. After the first flash: ●, Cyt *f*; ▲, Cyt *b*. After the second flash: ○, Cyt *f*, △, Cyt *b*. The maximum amount of photo-oxidable Cyt *f* corresponds to $\Delta I/I = 2.54 \cdot 10^{-3}$ (see Material and Methods). The same base line is taken for the first and the second flashes (dark-adapted material).

cedes the reduction of Cyt *f*. The reduction of Cyt *b* is followed by an oxidation phase which lasts for several seconds. The half-time of phase b, which has been measured after one and two flashes in the absence of dicyclohexyl-18-*crown*-6 is close to 8 ms, i.e., shorter than the oxidation phase of Cyt *b*. The spectral changes measured after the second flash when a large fraction of Cyt *b* is oxidized by the first flash, resemble the ones measured after a first flash given under aerobic conditions (data not shown).

We want to stress that under anaerobic conditions the half-time for the oxidation of Cyt *b* measured after a nonsaturating flash could be 5 times longer than the half-time of phase b.

In order to estimate the maximum light-induced oxidation of Cyt *b*, mutant S52 was submitted to a strong continuous illumination. In the case of the batch used in the experiment of Figs. 8 and 9, a maximum amount of 1.55 molecules of Cyt *b* per Cyt *b/f* complex are oxidized after 1 s illumination. During the course of the illumination, the kinetics of both Cyt *b* and Cyt *f* oxidation are complex and will not be discussed here. Under flash or continuous illumination given under anaerobic conditions, we observed a large negative signal in the green region of the spectrum which might be related to the reduction of secondary PS I acceptors.

Discussion

The results reported here raise numerous questions, some of which we are not able to answer on the basis of the classic models which describe the electron-transfer process in the Cyt *b/f* complex.

(1) In quinone-treated algae, electrons which are transferred to PS I all originate from plastoquinol formed through the two electron gate mechanism occurring on the acceptor side of PS II. On this material, the electrons transferred to any 'non-B' type acceptors [29] as Q_2 [30] or 'B' [31] are not available for PS I. For instance, we do not observe any phase b after one flash when the secondary acceptor Q_B has been fully oxidized by ferricyanide plus DAD.

(2) The number of electrons formed on the PS II acceptor side and which can be transferred to PS I donors is close to the number of holes formed on the PS I donor side (see Figs. 5 and 6). This conclusion agrees with the fact that the amplitudes of phase a associated with each of the two photoreactions are equal. The small difference in the absorption level measured 6 s after two flashes compared to the base line could arise from the 'misses' in PS II reaction [32], which are likely linked to an imperfect charge stabilization. The absence of a long-term storage of electrons in any of the carriers of the Cyt *b/f* complex is also demonstrated by the fact that the oscillations reported in Fig. 2 strictly reflect the oscillations due to the two-electron gate mechanism on the acceptor side of PS II. One can also exclude a long-term electron storage in benzoquinone which would have not been removed by washing.

The half-time of phase b is independent of the amount of plastoquinol formed after each flash of the series. This suggests that phase b and the intersystem electron transfer do not involve any type of cooperative phenomenon between Cyt *b/f* complexes or second-order processes such as for instance, sequential oxidation of two plastoquinols by the same Cyt *b/f* complex.

(3) In quinone-treated algae, the slow electron transfer from PS II to PS I under flash illumination does not seem to be due to irreversible damages induced by the quinone treatment or inhibitory effect due to quinone nonremoved by washing as proved by the following arguments. (a) After a group of two flashes, a similar rate of PS II donor reduction is measured both in quinone-untreated isolated spinach chloroplasts [13] and in quinone-treated algae. (b) In isolated spinach chloroplasts, the liberation of protons inside the thylakoid associated with the intersystem electron transfer lasts for several hundreds milliseconds [5]. (c) Under steady-state continuous illumination, a fast rate of electron transfer is observed (more than one electron every 30 ms). (d) After incubation in the presence of dithionite, the half-time of phase b is as short as 2.5 ms.

(4) in *Chlorella sorokiniana* as in higher plants, PS II centers are mostly located in stacked regions of large dimensions, while PS I is likely located in nonstacked regions; no reliable information is actually available on the location of Cyt *b/f* complexes in this material. The absence of a lag longer than 1 ms in the reduction of Cyt *b* (Fig. 6) or in

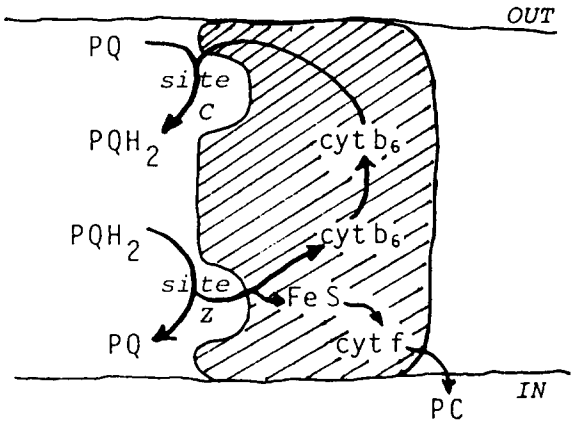
phase b (Fig. 3) shows that either a large proportion of Cyt *b/f* complexes is located close to PS II centers in the appressed region or the diffusion of plastoquinol from appressed to nonappressed regions occurs in less than 1 ms. This last hypothesis does not agree with the estimated diffusion time of plastoquinol given by Crofts and Wraight [33]. In any case, the diffusion of plastoquinol from PS II to Cyt *b/f* complex does not appear as a rate-limiting process in the intersystem electron transfer. It then remains difficult to understand why the half-time of phase b is 10–20 times slower when plastoquinol is formed by PS II reaction rather than chemically or by the respiratory pathway.

(5) In quinone-treated algae, the reduction of Cyt *b* is much faster than that of PS I donors (Fig. 6). Since this experiment is performed in the presence of an efficient PS I electron acceptor as ferricyanide, one can exclude that a cyclic electron flow around PS I participates to the reduction of Cyt *b*. On untreated algae, we also observed that the reduction of Cyt *b* widely precedes the reduction of Cyt *f* both under aerobic conditions (data not shown) and anaerobic conditions (second flash, Figs. 8 and 9). In isolated chloroplasts, Selak and Whitmarsh [34] arrived to similar conclusions, but their measurements were performed in the presence of HQNO which is known to modify the redox potential of Cyt *b* [35]. If we consider as valid the concept of a concerted reduction of Fe-S protein and Cyt *b* by plastoquinol, we must then assume that the transfer of one electron from Fe-S to Cyt *f* is a time-limiting process. This hypothesis is in contradiction with the fact that under reducing conditions, we do not observe a significant lag in the course of phase b or in the reduction of Cyt *b* which implies that a positive charge can be transferred from P-700 to the Fe-S center in less than a few hundred μs. We will discuss later a model which could solve this apparent contradiction.

Models

A phase b of large amplitude is observed when most of Cyt *b* and all the plastoquinones have been reduced in the dark by the anaerobic incubation. Girvin and Cramer [16] already observed a normal phase b in isolated chloroplasts under highly reducing conditions (−100 mV). Contrary to these authors, we observed a large flash-induced oxidation of Cyt *b*, as well in living algae (Fig. 9) as in isolated spinach chloroplasts in the presence of dithionite (data not shown). This difference might be due to the use by Girvin and Cramer of mediators which could rapidly rereduce Cyt *b* after the flash. In any case, both experiments cannot be interpreted in the frame of the classic Q-cycle model. In this class of models, the slow electrogenic phase is associated with a transmembrane electron transfer via the two Cyt *b*, which leads to the reduction of a plastoquinone on a site C located close to the outer face of the membrane (Scheme I). Under reducing conditions, no oxidized plastoquinone should be present at this site. The only available oxidant is the semiquinone which has been formed at size Z by the transfer of one electron to PS I donors. This semiquinone is very likely involved in the oxidation of reduced Cyt *b*. It is worthy to note that such a reaction is a part of the *b*-cycle model proposed by Wikström and Krab [11]. Nevertheless, since under anaerobic conditions phase b can be much faster than the oxidation of Cyt *b*, it is unlikely that a *b*-cycle process is obligatorily involved in the slow electrogenic phase.

In agreement with Girvin and Cramer [16], we think that the most likely hypothesis is that the transfer of electrons from plastoquinol to Cyt *f* is coupled to an electrogenic process which involves a proton-pumping device. Wikström and Krab [11] already proposed models in which the Cyt *b/f*



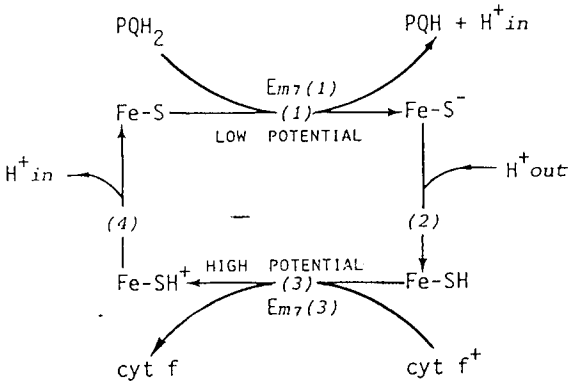
Scheme I

complex includes a true proton-pumping device which implies the presence of transmembrane proton channels. According to Papa et al. [12], the Fe-S center should be a good candidate to be coupled with this proton pump. In this type of models, a redox couple can exist in protonated and deprotonated forms with corresponding high and low redox potentials, respectively (Scheme II). Reaction 2 implies the electrogenic pumping of one proton from the external medium. A mobile structural device is required to explain that during Reaction 2, protons are exchanged only with the external aqueous phase, and that during Reaction 4, proton exchange occurs with the internal aqueous phase.

After a group of two flashes given to dark-adapted quinone-treated algae, the Fe-S center is already in its oxidized state. The reaction is triggered by the formation of plastoquinol by PS II. The process starts by the transfer of one electron from plastoquinol to the Fe-S center according to Reaction 1; then, through Reactions 2–4, the oxidized form of the Fe-S center is regenerated. The semiquinone formed by Reaction 1 transfers one electron to Cyt b^+ according to Reaction 5:

$$PQH + \text{Cyt } b^+ \rightleftharpoons PQ + \text{Cyt } b + H^+_{in} \qquad (5)$$

The redox potential of the couple PQH_2/PQH must be equal to or lower than +280 mV, potential of the couple Fe-S/Fe-S$^-$ [36] and the one of the couple PQH/PQ equal to or lower than the potential of Cyt b (−75 mV? [16]). The mean value must be equal to the potential of the couple PQH_2/PQ (approx. 100 mV).



Scheme II (Reactions 1–4)

The key point in our model is to assume that the time-limiting step is Reaction (2) with which is associated the electrogenic pumping of one proton. This hypothesis takes into account the fact that Cyt f reduction is slower than that of Cyt b. Two types of mechanisms nonmutually exclusive could be involved in the reoxidation of Cyt b: (a) a Q-cycle process which leads to the reduction of a plastoquinone located at site C; (b) if the semiquinone PQH can transfer an electron to PS I donors through Reaction (6), Cyt b could transfer electrons to PS I donors via Reaction (5), which is supposed to be close to equilibrium:

$$PQH + \text{Fe-S} \rightarrow PQ + \text{Fe-S}^- + H^+_{in} \qquad (6)$$

Rich [37] already proposed that under conditions in which the Q-cycle is not operating, the two electrons of plastoquinol could be sequentially transferred to PS I donors. As the redox potential of Cyt b is lower than that of PS I donors, the electrons would be slowly transferred from Cyt b to PS I donors.

Under reducing conditions, the Fe-S protein is in a reduced and protonated form Fe-SH. After a flash, Fe-SH is rapidly oxidized by PS I donors. Reactions (3), (4) and (1) must be completed in a time equal to or shorter than the lag time (less than 300 μs) of the electrogenic phase (Fig. 7). Then, reduced Cyt b transfers one electron to PQH, according to the following reaction:

$$\text{Cyt } b + PQH + H^+ \rightarrow PQH_2 + \text{Cyt } b^+ \qquad (7)$$

In this model, the amplitude and the kinetics of the electrogenic phase (Reaction (2)) do not depend upon the state of oxidation of Cyt b. Moreover, the oxidation of Cyt b is not obligatorily correlated with the slow electrogenic phase. It is likely that Reaction 7 is slower than Reaction 2, which explains why the oxidation of Cyt b lasts a longer time than phase b.

Our model does not explain why under anaerobic conditions, the amplitude of phase b can largely exceed the amplitude of phase a; for instance, after a nonsaturating flash, we observed that 1.4 charges are transferred via the slow elec-

trogenic process when one positive charge is transferred to PS I donors. This result suggests that more than one electron carrier of the Cyt *b/f* complex can be protonated via a transmembrane process. Among the possible candidates are the Fe-S center, Cyt *f* or plastoquinol as in Reaction 7.

We must stress that the arguments discussed here do not exclude that a Q-cycle mechanism operates under other experimental conditions, for instance when oxidized plastoquinone is present at site C and when more than one plastoquinol per Cyt *b/f* complex is available.

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