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Proton pumping and electron transfer in the cytochrome *b/f* complex of algae

Pierre Joliot and Anne Joliot

Institut de Biologie Physico-Chimique, 13, rue Pierre et Marie Curie, 75005 Paris (France)

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The electron-transfer reactions and the slow transmembrane electrogenic phase (phase b) have been studied in Photosystem-II-depleted mutant strains of *Chlorella sorokiniana* under anaerobic conditions. The dark reduction of cytochrome *b* previously oxidized by a strong illumination is a biphasic process. The first phase, completed in about 20 s, is associated with the reduction of the high-potential form of cytochrome *b*; a slower phase, completed in about 10 min, is associated with the reduction of the low-potential form of cytochrome *b*. The α-bands of these two forms differ slightly. Under repetitive weak flash illumination, i.e., when a fraction of cytochrome *b* is oxidized prior to each flash, phase *b* is associated with minor changes in cytochrome *b* redox changes. In the presence of 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide (NQNO), a large reduction of cytochrome *b* ($t_{1/2} \approx 2$ ms) is followed by a slow reoxidation. Under these conditions, phase *b* becomes biphasic, a first phase, insensitive to the inhibitor, is associated with cytochrome *b* reduction while the second phase, dependent upon the inhibitor concentration, is related to cytochrome *b* oxidation. These results are interpreted in terms of a modified Q-cycle model. A single weak flash given to dark-adapted algae, i.e., when the two *b* cytochromes are in their reduced state, induces a large cytochrome *b* oxidation with which is associated a phase *b* of longer duration but larger amplitude than the one observed under repetitive-flash illumination. Addition of 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide inhibits neither the oxidation of cytochrome *b* nor phase b. These results suggest that a proton pump is coupled to the redox changes of plastoquinone occurring at a site, Z, close to the inner face of the membrane. This model implies that site Z is connected to the outer face of the thylakoid by a proton channel. Thus, depending upon the experimental conditions, two different mechanisms might be involved in the process of proton-pumping by the cytochrome *b/f* complex.

Introduction

The transmembrane cytochrome *b/f* (Cyt *b/f*) complex is known to catalyze the electron transfer from plastoquinol to plastocyanin, the PS I secondary donor. It is generally assumed that a proton uptake on the outer face and a proton release on the inner face are associated with the electron transfer reactions which occur in the Cyt *b/f* complex. In living cells, or in isolated chloroplasts under reducing conditions, a short flash induces first a fast increase of the membrane potential (phase a, $t_{1/2} < 1$ μs) which is associated with the transmembrane photochemical charge separation and then a slow increase in the millisecond range (phase b) [1–3]. This slow electrogenic phase is

Abbreviations: PS, Photosystem; PQ, plastoquinone; PQH_2, plastoquinol; PQ^-, plastosemiquinone; cytochrome *b* or Cyt *b*, cytochrome b_6; Cyt *f*, cytochrome *f*; HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; NQNO, 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide.

associated either with a proton transfer from the outside to the inside of the thylakoid or with an electron transfer in the opposite direction.

In all models proposed to explain the mechanism of electron transfer in the Cyt *b/f* complex, plastoquinol is supposed to be oxidized at a specific site Z of the complex. A first electron is transferred to a high-potential electron transfer chain, which includes the FeS center and Cyt *f*, and the second electron is transferred to a low-potential chain including the two *b* cytochromes [4]. Following this first common step, several classes of model are proposed for the mechanism of proton-pumping and Cyt *b* re-oxidation.

(1) The more popular model derives from the original Q-cycle model of Mitchell [5]: in its modified version proposed by Crofts et al. [6], the two *b* cytochromes take part in an electrogenic loop which establishes a link between two sites, Z and C, able to fix plastoquinol or plastoquinone. Sites Z and C are close to the inner and the outer faces of the membrane, respectively. In this model the electrogenic phase is associated with electron transfer from site Z to site C.

(2) In a second class of model, a proton-pumping device is coupled to the redox changes of one of the electron carriers included in the Cyt *b/f* complex. In this class of model, the electrogenic phase is associated with a proton movement from the outer phase to the inner phase of the membrane. In the original form of the b-cycle, Wikström and Krab [7] proposed that the proton pump is coupled to the redox changes of Cyt *b*. Girvin and Cramer [8], to interpret their data obtained in highly reducing conditions, proposed that the proton pump is coupled to the electron transfer through the high-potential electron transfer chain, very likely at the level of the FeS center. Our recent data obtained with living algae [9] brought some support to this hypothesis, but we did not exclude the possibility that the high-potential carrier could be the couple PQH_2/PQ^- itself.

In this paper we present some data which establish that proton-pumping is associated with Cyt *b* turnover, even under reducing conditions. Depending upon the state of oxidation of Cyt *b* prior to a flash illumination, two different processes of proton-pumping are involved.

Material and Methods

The experiments were performed with two mutant strains of *Chlorella sorokiniana* which lack PS II. These mutants were characterized from biochemical, structural [10] and biophysical studies [11]. The S52 strain presents a very small pigment antenna and is thus well-adapted to the measurement of Cyt *b* spectral changes. On the other hand, the low content in electrochromic probes makes the measurement of the membrane potential more difficult. The S8 strain, which has a normal antenna size, is preferentially used for the measurement of membrane potential. The experiments are performed at room temperature in 0.05 M phosphate buffer (pH 6.5)/10% (v/v) Ficoll. The reducing conditions were obtained by incubation of the algae in the measuring cuvette for more than 20 min in the presence of 20 mM glucose plus 3 mg/ml glucose oxidase.

Spectrophotometric measurements were performed with an apparatus similar to that described in Ref. 12 and improved according to Ref. 13. Actinic excitation is provided either by a xenon flash (3 μs duration at half-height), filtered through a Schott filter (RG5 or RG8), or by a dye laser flash Candela SLL150. The emission peaks at 692 nm (oxazine) and the total duration of the flash (about 700 ns) is short enough not to induce double photoreactions.

The variations of the membrane potential are followed by the electrochromic shift of the membrane pigments. The field-indicating absorption change was measured by the difference $\Delta I/I$(514 nm) $- \Delta I/I$(474 nm).

For the comparison between Cyt *f* and Cyt *b* contents, the extinction coefficient for Cyt *f* was derived from Ref. 14 and from Ref. 15 for Cyt *b*.

Under reducing conditions and for detecting times longer than 50 μs, spectral changes due to PS I primary and secondary donors are generally small and do not perturb Cyt *b* measurement in the α-band.

Results

As shown by Bennoun [16], chloroplasts of living cells include a respiratory pathway which feeds in the plastoquinone pool with electrons: the

plastoquinone pool is reoxidized by a specific oxidase. Measurement of the fluorescence induction curves in wild-type algae shows that anaerobic incubation rapidly induces the total reduction of the plastoquinone pool. Long anaerobic incubation (over 20 min) induces the reduction of a large fraction of Cyt *b*. Then, illumination by a strong continuous light oxidizes more than 1.6 Cyt *b* molecules per Cyt *b/f* complex, calculated on the basis of the Cyt *f* content [9]. In a subsequent dark period, the re-reduction of Cyt *b* is markedly biphasic, a fast phase which concerns half of the total amplitude and completed in about 20 s is followed by a slower phase which lasts for more than 10 min (data not shown). These results suggest that there are two types of Cyt *b* which can differ either by their membrane environment or by their redox potential. Titrations performed in chloroplasts [17–19] lead to very different values for the midpoint potential of Cyt *b* (between +5 mV and −140 mV). Recently, Girvin and Cramer [8] measured a midpoint potential of about −30 mV for both cytochromes *b*. Titrations performed in isolated Cyt *b/f* complexes appear more reliable: Hurt and Hauska [20,21] and Clark and Hind [22] observed two waves in Cyt *b* titration with midpoint potentials around −30 mV and −160 mV. The simplest interpretation of our data is to suppose that in living algae, as in isolated Cyt *b/f* complexes, there are two forms of Cyt *b*, a high-potential form rapidly reduced and a low-potential form slowly reduced.

Slow electrogenic phase under single or repetitive-flash illumination

In Fig. 1, algae were illuminated by a single non-saturating flash following 20 min dark-adaptation (curve 1) or by a flash series 6 s apart (curve 2). Curve 1 shows that the decay of the membrane potentials is slow under anaerobic conditions ($t_{1/2}$ ≈ 3 s). Depending upon the algal culture, the amplitude of the slow electrogenic phase after a long dark adaptation can vary from 1.2 to 1.5 charges transferred through the membrane per positive charge formed by the PS I reaction. This amplitude is generally larger than the one measured after repetitive-flash illumination. Phase b is about 2-times slower after a single flash than under repetitive-flash illumination or when a flash

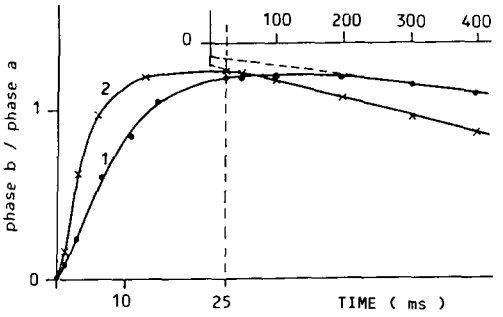


Fig. 1. Phase b induced by a non-saturating flash (hitting 30% of the PS I centers). S8 mutant strain in anaerobic conditions. Curve 1, single-flash illumination after 20 min dark-adaptation. Curve 2, repetitive flashes 6 s apart. The amplitude of phase a was identical for both curves.

is given under aerobic conditions (data not shown). Depending on the algal culture or on the mutant strain, notable variations in the half-time of phase b can be found.

Flash-induced Cyt b and Cyt f oxidations

In Fig. 2, algae dark-adapted for more than 10 min (curve 1) or for 20 s (curve 2) were submitted to a single-flash illumination of various energy and duration. The amplitude of Cyt *b* oxidation is plotted as a function of the number of positive charges formed by PS I reaction which is proportional to the amplitude of phase a. If we assume

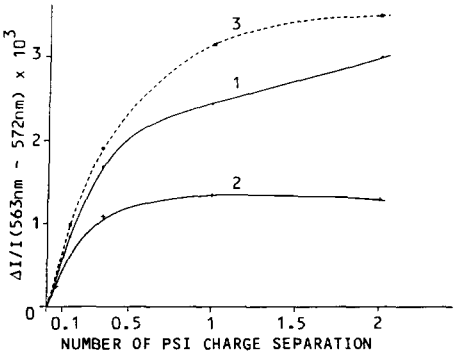


Fig. 2. Amplitude of the flash-induced oxidation (solid lines) or reduction (dashed line) of Cyt *b* as a function of the amplitude of phase a measured 200 μs after the flash and normalized to 1 for a saturating laser flash. S52 mutant strain. Curve 1, algae dark adapted for more than 10 min. Curve 2, 20 s dark adaptation following a continuous illumination. Curve 3, Cyt *b* reduction under repetitive-flash illumination 6 s apart in the presence of 2 μM NONO.

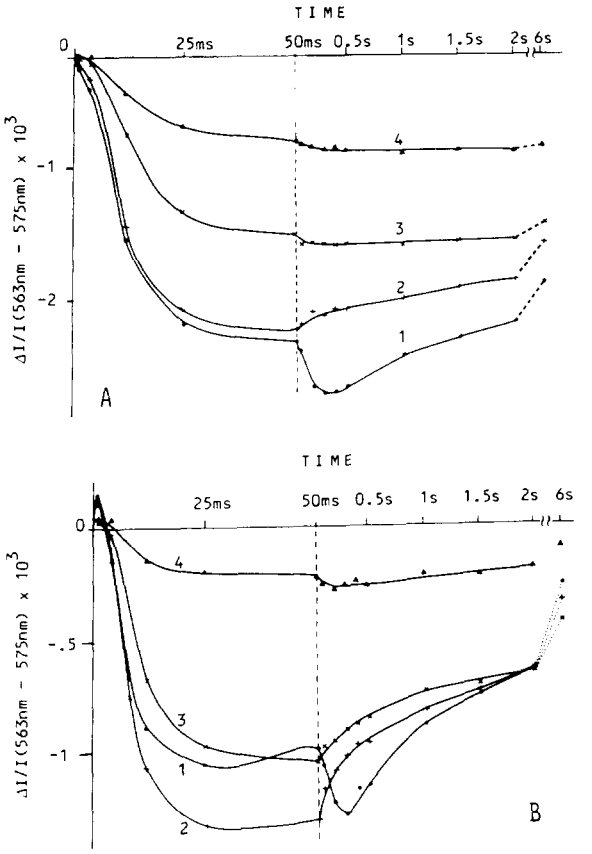


Fig. 3. Time-course of the redox changes of Cyt *b* after a flash of different energy and duration. S52 mutant strain. (A) Algae dark-adapted for more than 10 min. (B) Algae dark-adapted for 20 s after 2 s strong continuous illumination. Curves 1, high-energy xenon flash. Curves 2, saturating laser flash. Curves 3, xenon flash hitting 34% of PS I centers. Curve 4 of part A, xenon flash hitting 15% of PS I centers. Curve 4 of part B, xenon flash hitting 6% of PS I centers.

that a saturating laser flash induces one charge separation per PS I center, the strongest xenon flash induces about two charge separations due to a large probability of double hits. Curves 1 and 2 show that the ratio between the number of photo-oxidized Cyt *b* and the number of positive charges available among PS I donors increases when the number of positive charges decreases. The initial slopes of curves 1 and 2 are almost equal, while the amount of Cyt *b* oxidized by a strong flash is 2–3-times higher in dark-adapted than in preilluminated material.

Fig. 3A and B shows the flash-induced kinetics of Cyt *b* redox changes in dark-adapted and preilluminated material, respectively, under the same conditions as in Fig. 2. All these kinetics display a lag of about 5 ms duration in the course of Cyt *b* oxidation; this lag is either an actual delay in the oxidation of Cyt *b* or the result of the superimposition of a fast reduction phase of Cyt *b*. For a weak flash, the overall half-time for Cyt *b* oxidation is 15 ms in dark-adapted material and 11 ms in preilluminated material. An essential feature concerns the rate of re-reduction of Cyt *b*: after a weak flash given to dark-adapted material, the redox state of Cyt *b* remains stable for several seconds, while a significant re-reduction is observed in the same time range for stronger intensity of the actinic flash. On the other hand, in preilluminated material, a fast re-reduction of Cyt *b* is observed even for weak flashes. In the presence of dicyclohexyl-18-*crown*-6, which is known to collapse the membrane potential in vivo (Diner, personal communication), the kinetics of Cyt *b* re-reduction are significantly slowed down (data not shown).

In Fig. 4 are plotted the absorption changes associated with the reduction of Cyt *b* previously oxidized by a strong xenon flash. The spectrum of Cyt *b*, which is rapidly reduced (240 ms–10 s) significantly and reproducibly differs from the

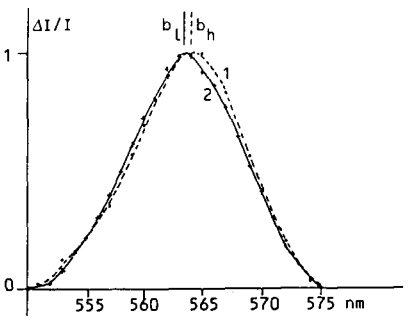


Fig. 4. Reduced minus oxidized spectra of Cyt *b*. S52 mutant were illuminated by a strong xenon flash which induces the oxidation of a major fraction of Cyt *b* in less than 100 ms. Spectrum 1, absorption changes (240 ms–10 s) after the flash. Spectrum 2, absorption changes (20 s–30 min) after the flash. The two spectra were compared by taking as a baseline a straight line connecting the absorption changes at 550 nm and 575 nm. Before normalization of the spectra, the absolute amplitudes were $\Delta I/I = 1.34 \cdot 10^{-3}$ and $1.71 \cdot 10^{-3}$ for spectra 1 and 2, respectively.

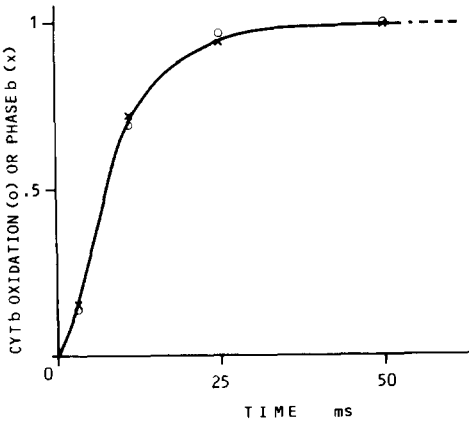


Fig. 5. Time-course of Cyt *b* oxidation and phase b induced by a single laser flash illumination. S52 mutant strain dark-adapted for about 11 min under anaerobic conditions. Both kinetics were measured on the same batch and were normalized to their maximum amplitudes: $\Delta I/I(563\text{ nm}-575\text{ nm}) = 2.6 \cdot 10^{-3}$ for Cyt *b* and $\Delta I/I(514\text{ nm}-474\text{ nm}) = 4.2 \cdot 10^{-3}$ for phase b. Phase b has been deconvoluted from the membrane potential decay.

spectrum of Cyt *b* slowly reduced (20 s–30 min).

Cyt *f* redox changes have been measured under repetitive-flash illumination. 1 ms after each flash, no more than 0.1 molecule of Cyt *f* is oxidized per positive charge formed by PS I. The half-time for its re-reduction is shorter after a weak flash ($t_{1/2} \simeq 3$ ms) than after a short saturating flash ($t_{1/2} \simeq 7$ ms) (data not shown). As we will discuss in more detail later, these data suggest that after strong flashes, more than 1 positive charge can be sequentially transferred to the same Cyt *b/f* complex. For this reason, we chose in the following experiments to use exclusively non-saturating flashes with which the probability for a complex to receive more than one positive charge is negligible.

Correlation between Cyt b oxidation and the slow electrogenic phase

The time-course of flash-induced Cyt *b* oxidation after a long dark anaerobic incubation can significantly vary depending on the culture or on the mutant strain (compare Figs. 3A, 5 and 9A). A similar variability is observed in the kinetics of phase b. In Fig. 5 are shown the time-course of both Cyt *b* oxidation and phase b kinetics measured on the same batch. After a long dark anaerobic incubation, the kinetics of both phenomena are identical. After a shorter period of dark adaptation, i.e., when a fraction of Cyt *b* remains in its oxidized form, the half-time of the slow electrogenic phase is always shorter than that of Cyt *b* oxidation.

Effect of NQNO

Repetitive-flash illumination. HQNO and NQNO are known to block Cyt *b* oxidation in Cyt *b/f* complexes [23,24]. For the lowest concentrations of NQNO (0.1 and 0.5 μM), an incubation time of about 30 min is required to reach a steady-state level of inhibition, which then remains stable for

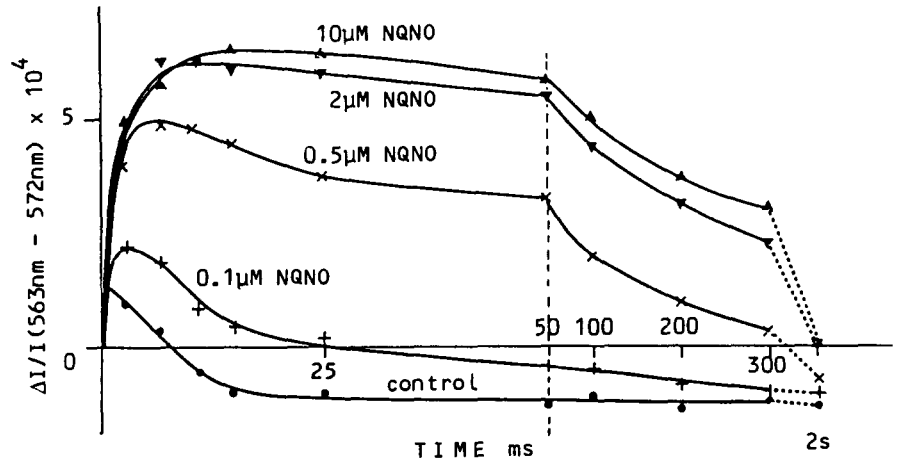


Fig. 6. Time-course of the flash-induced redox changes of Cyt *b* in the presence of various concentrations of NQNO. S8 mutant strain. Repetitive nonsaturating flashes 8.6 s apart hitting 39% of PS I centers.

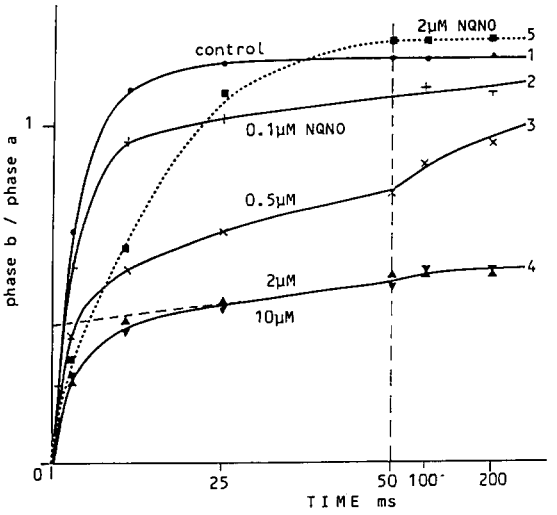


Fig. 7. Time-course of phase b in the presence of various concentrations of NQNO. Solid lines: same conditions as in Fig. 6. Dashed line: single-flash illumination after 30 min dark-adaptation in the presence of 2 μM NQNO. Phase b has been deconvoluted from the decay of the membrane potential.

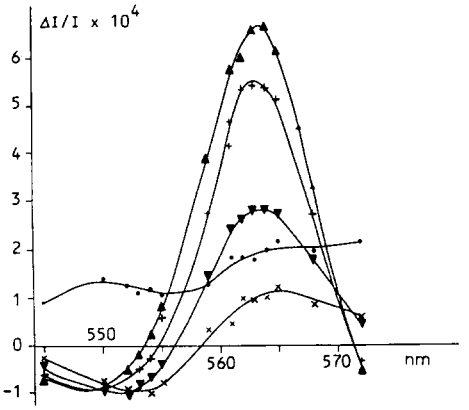


Fig. 8. Flash-induced absorption changes in the presence of 2 μM NQNO. S52 mutant strain. Repetitive non-saturating flashes 3 s apart hitting 12% of PS I centers. ●, 60 μs; ×, 300 μs; ▼, 1 ms; +, 3 ms; ▲, 11 ms.

several hours. For higher NQNO concentrations, a much shorter incubation time is required.

A first effect of NQNO is to slow down the reduction of Cyt *b* during the dark adaptation. Consequently, it is often impossible to reduce Cyt *b* totally, even after dark-adaptation longer than 30 min.

The time-courses of Cyt *b* redox changes (Fig. 6) and of phase *b* (Fig. 7) upon illumination by repetitive non-saturating flashes 8.6 s apart have been measured in the presence of various concentrations of NQNO. In the absence of the inhibitor, phase b corresponds to the transfer of 1.2 charges per positive charge formed by the PS I reaction. Cyt *b* redox changes are small, which is very likely due to the overlapping of reduction and oxidation phases. Addition of NQNO does not inhibit Cyt *b* reduction but markedly slows down its oxidation. The half-inhibition concentration is about 0.2 μM. In the presence of NQNO, phase b becomes biphasic: a fist phase, NQNO-insensitive, follows the time-course of Cyt *b* reduction and corresponds to the transfer of 0.4 charges per positive charge formed by PS I (extrapolation to time zero of curve 4, Fig. 7); the time-course of the second phase follows approximately the time-course of Cyt *b* oxidation and is thus NQNO-sensitive. A secondary effect of NQNO is to accelerate the decay of the membrane potential ($t_{1/2} \approx$ 500 ms for 2 μM NQNO). We want to stress that it is difficult to compare precisely the time-course of Cyt *b* oxidation with that of the slowest part of phase b: during the first 50 ms following the flash, Cyt *b* oxidation and reduction overlap; on the other hand, the slowest part of phase b cannot be accurately deconvoluted from the decay of the membrane potential, as these two processes occur in the same time-range.

Fig. 8 shows the spectral changes in the 545 nm–572 nm range induced by repetitive weak flashes 3 s apart in the presence of NQNO. Beyond 300 μs, the amplitude of the signals which can be ascribed to the oxidation of PS I primary and secondary donors (P-700, plastocyanin and Cyt *f*) is small when compared to the maximum extent of Cyt *b* reduction. Then, it is very likely that 300 μs after a weak flash most of the positive charges formed by PS I are located on the FeS center, the redox changes of which are not easily detectable. A larger relative amplitude of Cyt *f* oxidation would be observed after a saturating flash.

In the experiment of Fig. 2, curve 3, we measured in the presence of 2 μM NQNO and under repetitive-flash illumination the maximum amount of Cyt *b* reduced by flashes of different energy and duration. As for the oxidation of Cyt *b* in the absence of inhibitor (curves 1 and 2), the amount

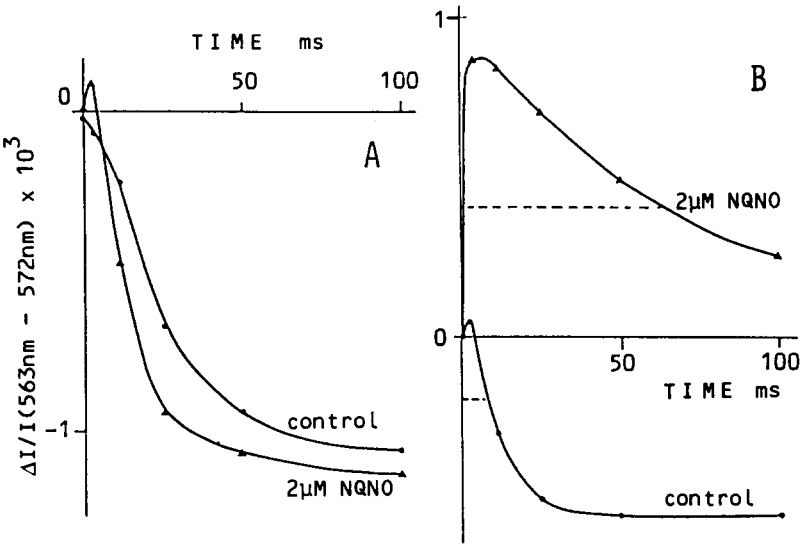


Fig. 9. Time-course of the flash-induced redox changes of Cyt *b*. S52 mutant strain. Non-saturating flashes hitting 45% of the PS I centers. (A) single-flash illumination after 30 min dark-adaptation. (B) Repetitive flashes 5 s apart.

of Cyt *b* reduced is nonlinearly related to the number of positive charges formed per PS I charge separation. The initial slopes of the three curves are similar and very likely correspond to the oxidation (curves 1 and 2) or the reduction (curve 3) of one Cyt *b* molecule per positive charge formed by PS I reaction.

Single-flash illumination. The flash-induced oxidation of Cyt *b* was studied using the S52 mutant dark-adapted for 30 min in the presence of a saturating concentration of NQNO (Fig. 9A). Surprisingly enough, under these conditions, not only does NQNO not inhibit Cyt *b* oxidation, it slightly accelerates it. Under the same experimental conditions, the amplitude of phase b is not inhibited by NQNO (data not shown). The experiment of Fig. 9A was performed after experiment Fig. 9B, in which we checked that in preilluminated material, NQNO actually induces a large inhibition of Cyt *b* oxidation.

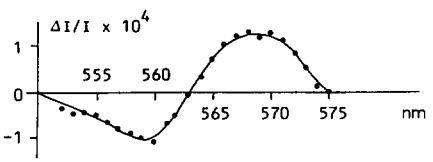


Fig. 10. Effect of 2 μM NQNO on the spectrum of reduced Cyt *b*. Difference between the spectra measured in the dark in the presence and in the absence of NQNO. In both cases, algae were dark adapted for 30 min under anaerobic conditions.

The slow electrogenic rise in the presence of NQNO and after a long dark adaptation was measured with a better accuracy on mutant S8 (Fig. 7, curve 5). As already observed with mutant S52, NQNO does not inhibit phase b in this dark-adapted material. The half-rise time (about 11 ms) is the same as that of Cyt *b* oxidation measured in the presence of NQNO (Fig. 9A).

We already pointed out that it is often difficult to reduce Cyt *b* totally in the dark in the presence of NQNO. In this case, the kinetics of Cyt *b* oxidation under single flash-illumination are biphasic.

Fig. 10 shows the difference between the spectra measured in the dark with algae dark-adapted for more than 30 min in the presence or in the absence of NQNO. This difference spectrum shows that NQNO induces a red shift of 0.2–0.3 nm of the α-band of Cyt *b*.

Discussion

The hypothesis we suggested in the previous section that dark anaerobic incubation induces the sequential reduction of a high potential Cyt b_h and a low potential Cyt b_l is crucial for the

interpretation of our data and is supported by several arguments: (a) illumination by a strong continuous light of dark-adapted material induces the oxidation of more than one molecule of Cyt *b* per Cyt *b/f* complex (measured on the basis of the total Cyt *f* content), which implies that more that one Cyt *b* molecule was reduced prior to the illumination. (b) Clark and Hind [22] already reported that dithionite induced a biphasic reduction in the second and minute ranges of Cyt *b* in isolated *b/f* complex of spinach chloroplasts. They interpreted these data by a sequential reduction of Cyt b_h and Cyt b_l, a process which resembles the one we observe in living algae in anaerobic conditions. (c) Low-temperature spectra obtained by Hurt and Hauska [21] show that Cyt b_h displays two peaks (557.5 nm and 561.5 nm) and Cyt b_l a single peak at 560.5 nm. Our data qualitatively agree with these spectra, taking into account that low temperature shifts the spectrum about 3 nm towards shorter wavelengths and decreases the bandwidth. We actually observe (Fig. 4) that Cyt b_l peaks at slightly shorter wavelength than Cyt b_h. On the other hand, the larger bandwidth we observe for Cyt b_h could be due to the superimposition of two peaks, unresolved at room temperature.

Electron transfer reactions

The first electron transfer reactions which follow a flash given under reducing conditions are the transfer of a positive charge from P-700 to a high-potential carrier of the Cyt *b/f* complex and the oxidation of plastoquinol PQH_2 in plastosemiquinone PQ^-, according to the reaction 0:

$$PQH_2 + (\text{FeS Cyt } f)^+ \rightarrow PQ^- + (\text{FeS Cyt } f) + 2H^+ \qquad (0)$$

The following steps will depend upon the redox state of the two *b* cytochromes included in the complex. The three possible states of the Cyt *b/f* complex are $(b_l b_h)$. $(b_l^+ b_h)$ and $(b_l^+ b_h^+)$. Depending upon the initial state, the following reactions will occur:

$$(b_l b_h) + PQ^- + 2H^+ \rightarrow (b_l^+ b_h) + PQH_2 \qquad (1)$$

$$(b_l^+ b_h) + PQ^- \xrightarrow{(2A)} (b_l b_h) + PQ \xrightarrow[2H^+]{(2B)} (b_l^+ b_h^+) + PQH_2 \qquad (2)$$

$$(b_l^+ b_h^+) + PQ^- \rightarrow (b_l^+ b_h) + PQ \qquad (3)$$

Moreover, the two *b* cytochromes become sequentially re-reduced in the dark, according to the reactions:

$$(b_l^+ b_h^+) \underset{5\text{–}10\ s}{\overset{(4A)}{\rightarrow}} (b_l^+ b_h) \underset{3\ min}{\overset{(4B)}{\rightarrow}} (b_l b_h) \qquad (4)$$

Reactions 0 to 4 do not imply any hypothesis, either on the number of sites involved in the plastoquinone fixation or on the mechanism of proton-pumping. As discussed below, these reactions may satisfactorily account for the flash-induced kinetics of oxidation and reduction of Cyt *b*.

On the basis of experiments shown in Figs. 2 and 3 we can distinguish the properties of states $(b_l b_h)$ and $(b_l^+ b_h)$: according to reaction 1, a weak flash given to dark-adapted material (initial state $b_l b_h$) induces the formation of Cyt b_l^+, which is slowly re-reduced by reaction 4B and remains stable in the 0–5 s time range (Fig. 3A, curve 4). After 20 s dark-adaptation (initial state $b_l^+ b_h$), the same weak flash induces the formation of an equivalent amount of Cyt b_h^+ (reaction 2), which is more rapidly reduced by reaction 4A (Fig. 3B, curve 4).

Saturation curves

Fig. 2 shows that the ratio between the number of photooxidized Cyt *b* and the number of positive charges formed by PS I reaction progressively decreases when the number of positive charges increases. This result shows that the electron transfer to Cyt *b/f* complex is not a first-order process, which is to be expected if one remembers that positive charges are transferred via a diffusible carrier, plastocyanin. Then, upon illumination by a strong flash, the distribution of positive charges among the Cyt *b/f* complexes could be unequal. As Cyt *b/f* complexes include two sites for positive charges (FeS and Cyt *f*), one can expect that upon illumination by a strong flash, the complexes can store 0, 1 or 2 of these charges. Moreover, if there is a large excess of positive charges, it is very likely that more than two positive charges could be sequentially transferred to a fraction of the complexes after the reduction of FeS by plastoquinol. The dependence of Cyt *f* oxidation upon the flash energy agrees with this assumption; for a weak flash, the probability of a

Cyt b/f complex's receiving more than one positive charge is negligible, which explains why 300 μs after the flash, most of the positive charges is stored on the FeS center and very little on Cyt f (Fig. 8). The decrease in the rate of Cyt f reduction we observed after a strong flash indicates that a second positive charge can be transferred to the same complex. We can now interpret the saturation curves of Fig. 2: when most of the complexes are in the $(b_l^+ b_h)$ state, the first positive charge transferred to a complex leads to the oxidation of Cyt b_h according to reactions 2, but the second positive charge would lead to the re-reduction of Cyt b_h^+ according to reaction 3. When a sufficient amount of positive charges is available, a maximum of 0.5 molecule of Cyt b would be oxidized, to which corresponds to an equipartition of the complexes between states $(b_l^+ b_h^+)$ and $(b_l^+ b_h)$. On the other hand, when most of the complexes are in the $(b_l b_h)$ state, the first positive charge leads to the oxidation of Cyt b_l and the second one to the oxidation of Cyt b_h: only the third positive charge transferred can lead to the re-reduction of Cyt b_h^+ (reaction 3). Then, after a strong and long flash, we expect the oxidation of a maximum amount of 1.5 Cyt b molecules. As expected, a fast reduction of Cyt b_h^+ is observed when a strong flash is given to complexes in the $(b_l b_h)$ state (Fig. 3A, curve 1). The relative amplitude of curves 1 and 2, Fig. 2, are in reasonable agreement with the prediction of our model. In order to fit the saturation curves quantitatively to our model, we need to assume that the Cyt b/f complexes are at least 2-times less concentrated than PS I centers. A possible hypothesis is that under strong flash- illumination, the Cyt b/f complexes located close to PS I centers trap the totality of the positive charges formed by PS I reaction. These Cyt b/f complexes would undergo several turnovers, while the complexes located at longer distance, for instance in the appressed region of the thylakoids [25], would not participate in the electron transfer reactions.

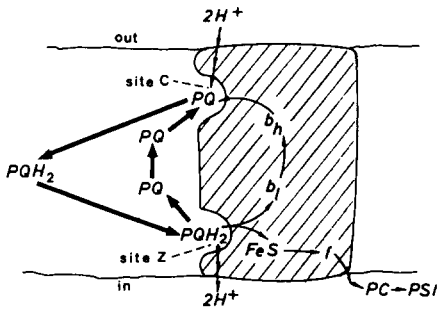
Effect of NQNO

Under repetitive-flash illumination, the complexes are distributed between $(b_l^+ b_h)$ and $(b_l^+ b_h^+)$ states, depending upon the time interval between flashes. Fig. 6 shows that the oxidation step (reaction 2B) is severely inhibited (by about a factor 50) by NQNO, while the Cyt b reduction (reactions 2A and 3) is not inhibited. For increasing concentrations of NQNO, we observe a slowing down of both Cyt b oxidation and of a fraction of phase b, which clearly demonstrates that these two processes are related.

After a long dark incubation, we do not observe any inhibitory effect of NQNO or HQNO either on Cyt b oxidation or on phase b. A possible hypothesis is that the affinity of NQNO for complexes in the $(b_l b_h)$ state is much lower than for the complexes in the $(b_l^+ b_h)$ state. Several arguments can be raised against this hypothesis: (a) in the isolated Cyt b/c complex, Kamensky et al. [26] reported that NQNO and antimycin induce a red shift of the α-band of Cyt b of 0.06 nm and 0.5 nm, respectively. We observed that in dark-adapted material, NQNO induces a red shift of about 0.2 to 0.3 nm, a value significantly larger than the one measured in Cyt b/c complex. It is then very likely that NQNO is associated with the Cyt b/f complex when the two b cytochromes are in their reduced states. (b) NQNO induces a slight acceleration of Cyt b oxidation. This result also suggests that the inhibitor is associated with the complex. (c) Clark and Hind [22] reported that the redox potentials of b_l and b_h are shifted toward higher potentials by addition of HQNO, which implies that in the isolated complex, the $(b_l b_h)$ state normally fixes HQNO.

Models

As already pointed out by Selak and Whitmarsh [23], the effects of HQNO and NQNO under repetitive-flash illumination are better interpreted in terms of a modified Q-cycle [6] (Scheme I). The fact that NQNO exclusively acts on the photooxidation and not on the photoreduction of Cyt b strongly favors a model in which the Cyt b/f complex includes two distinct sites able to fix PQ, a characteristic feature of the Q-cycle class of model. Nevertheless, some hypotheses should be added to the classic modified Q-cycle model. Under reducing conditions there is no available molecule of PQ in the pool; we must then assume that the PQ formed by reaction 2A at site Z must be rapidly (in less than a few milliseconds) transferred in the same complex to site C (able to fix NQNO), where reaction 2B occurs, it



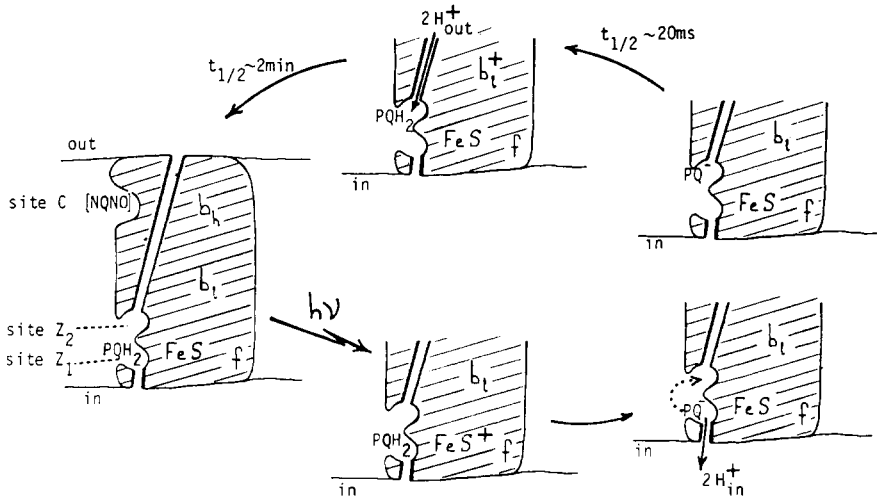
Scheme I.

is important to stress that, even in the presence of saturating concentrations of NQNO, i.e., when Cyt *b* oxidation is considerably slowed down, PQ formed at site Z which is the only available oxidant must remain attached to the complex for several hundred milliseconds until it is reduced by the two *b* cytochromes. The crucial point is that this PQ must not be reduced by the reducing pathway of the respiratory chain. On the contrary, when PQ is formed by reaction 3, it should rapidly escape from the complex to be replaced by a new molecule of PQH_2 to make the complex ready for a new turnover. This suggests that the interactions between PQ and the complex are controlled by the redox state of Cyt *b*. The saturation curve for Cyt *b* reduction in the presence of NQNO (Fig. 2, curve 3) can also be interpreted in terms of a Q-cycle model. Upon illumination by a weak flash, a reduction of Cyt *b* is expected for all complexes which receive a positive charge (reactions 2 and 3), which explains why the initial slope of curve 3 is equal to that of curves 1 and 2. After a strong flash, complexes in the $(b_l^+ b_h^+)$ state could undergo two turnovers and complexes in the $(b_l^+ b_h)$ state only one turnover; we then expect a maximum of 1.5 reduced Cyt *b* per Cyt *b/f* complex.

The dependence of phase b on NQNO concentration is also coherent with a modified Q-cycle model. In the absence of NQNO, the amplitude of phase b should be equal to one electron transferred through the membrane per positive charge formed by PS I; the measured value is slightly higher (1.2). In the presence of NQNO, the reduction of Cyt *b* should be associated with an increase in the membrane potential, which would depend upon the location of the two *b* cytochromes in the membrane. Recent structural studies [27,28] establish that the two hemes are about 20 Å apart and are located in a rather symmetric position with respect to the two faces of the membrane. For the complexes in the $(b_l^+ b_h)$ state, only Cyt b_l, which is located close to the inner face of the membrane, is reduced by the flash; for the complexes in the $(b_l^+ b_h^+)$ state, the flash induces the reduction of Cyt b_h, located close to the outer face. Then, in the average, phase b associated with Cyt *b* reduction would correspond to the transfer of one electron on the half of the membrane thickness, a prediction close to the measured value (0.4, see Fig. 7, extrapolation to time zero of curve 4). Contrary to Jones and Whitmarsh [24], we observe that, in the presence of NQNO, phase b and Cyt *b* reduction occur in the same time-range. Therefore, in the presence of NQNO, electron transfer between the two *b* cytochromes does not appear as a rate-limiting process as supposed by these authors.

After a long dark-adaptation, the photooxidation of Cyt *b* associated with a large phase b cannot be easily interpreted by a Q-cycle model. Under these conditions, PQ^- formed at site Z by reaction 0 cannot be oxidized into PQ, as the two *b* cytochromes are in the reduced state. Therefore, Cyt *b* must be oxidized by PQ^-, according to reaction 1. It is unlikely that cooperative interactions involving several complexes could lead to a dismutation of PQ^- into PQ and PQH_2, as the time-course of Cyt *b* oxidation is independent of the flash energy (first-order process). Recently, Wikström and Saraste [29] proposed that the semiquinone could move across the membrane from site Z to site C. In any case, a semiquinone – charged or even neutral – cannot move freely in a membrane without being destroyed by a dismutation reaction. Then one has to assume that the complex includes a specialized channel for PQ^-, termed 'Q-pocket' by Wikström and Saraste. This hypothesis does not explain why Cyt *b* is oxidized and phase b normally occurs when site C is occupied by NQNO.

We presently favor a model in which two different mechanisms could be involved in the process of proton-pumping. In repetitive-flash illumination and in the absence of NQNO, the Q-cycle model correctly describes our data. In



Scheme II.

single-flash experiment with or without inhibitor (dark-adapted material, $b_l b_h$ state), or under repetitive-flash illumination in the presence of NQNO, a b-cycle mechanism occurs which involves only site Z. In this model, reaction 0 releases protons inside the thylakoid while the protons involved in reaction 1 are picked upo on the outer face of the membrane. This model implies that a proton channel connects site Z to the outer face of the membrane and that a gate mechanism correctly orientates the proton flux. This gate mechanism could be linked to a small displacement of PQ^- from a site, Z1, connected to the FeS center to a site, Z2, connected to Cyt b_l (Scheme II). Proton channels oriented toward the inner and the outer faces would be connected to Z1 and Z2, respectively. In this respect, this model resembles the one proposed by Wikström and Saraste [29], except that PQ^- displacement occurs over a very short distance and not from site Z to site C.

The same mechanism for Cyt *b* oxidation could be involved in repetitive-flash illumination in the presence of NQNO, which occupies site C. The two electrons of Cyt *b* could be sequentially transferred to PQ which has been formed at site Z.

Our model does not explain why Girvin and Cramer [8] do not observe significant Cyt *b* photooxidation which spinach chloroplasts under highly reducing conditions. In the presence of dithionite but in the absence of any mediator, we do observe a large Cyt *b* oxidation in spinach chloroplasts: it is then possible that the mediator used by Girvin and Cramer rapidly re-reduces the oxidized Cyt *b*.

The main criticism against our hypothesis is that a proton-pumping process which would only operates after a single flash given in highly reducing conditions would be of no physiological use. The possibility is not excluded that a high value of the membrane potential which displaces the equilibrium constant of the electron transfer reaction between the two *b* cytochromes could favor the process of proton-pumping occurring at site Z. Another possibility is that this process is involved in cyclic electron transfer around PS I.

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