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The low-potential electron-transfer chain in the cytochrome *b/f* complex

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The oxidized-minus-reduced spectra of the low- and high-potential cytochromes b_6 were measured from 380 to 590 nm under anaerobic conditions in a mutant of *Chlorella sorokiniana* lacking of Photosystem II (PS II). The two spectra are similar in the Soret band; in the α-band, cytochrome b_l and cytochrome b_h peak at 563.4 and 564 nm, respectively. The spectrum of a third component, *G*, located on the outer face of the membrane and able to exchange electrons with cytochrome b_h (Lavergne, J. (1983) Biochim. Biophys. Acta 725, 25–33) was characterized. The oxidized-minus-reduced spectrum of *G* displays a negative band peaking around 424 nm (probably a double peak) and a broad and small negative band in the green region. This spectrum resembles that of a soluble high-spin cytochrome *c'*, as characterized in several classes of photosynthetic bacteria. As other cytochromes *c'*, G binds CO with a low affinity, which inhibits electron exchange with cytochrome b_h. The kinetics of reduction of cytochrome b_h and cytochrome b_l were measured in algae under anaerobic conditions, following illumination by weak continuous light or saturating flashes. From the kinetic behaviour, the difference between the midpoint potentials of cytochrome b_h and cytochrome b_l was estimated at approx. 140 mV. Two populations can be distinguished among cytochrome *b/f* complexes. In a first fraction, the complexes are associated with the carrier *G*, which very likely takes part in an electron-transfer chain mediating cytochrome *b* reduction by a stromal reductant. This chain could be involved in cyclic electron flow around Photosystem I. In the second fraction, where *G* is not attached to the complex, the reduction of the *b*-cytochromes is a much slower process. Cytochrome b_h and *G* are in equilibrium, the midpoint potential of *G* being approx. 20 mV higher than that of cytochrome b_h. From the study of the PS II-driven photoreduction of cytochrome b_h and *G* (under aerobic conditions) in a mutant which lacks of PS I, we conclude that the affinity of plastosemiquinone for site C is much higher than that of plastoquinol, which makes the properties of this site similar to those of PS II secondary acceptor Q_B. These results are discussed in terms of a model in which the semiquinone can rapidly move from site Z to site C.

Introduction

The cytochrome *b/f* complex is known to include four electron carriers, two of them (the Rieske FeS protein and cytochrome *f*) belong to a high-potential electron-transfer chain and are located close to the inner face of the membrane. The two other components (cytochrome b_h and cytochrome b_l) take part in a low-potential trans-

Abbreviations: PS, Photosystem; PQ, plastoquinone; PQH_2, plastoquinol; PQ^-, plastosemiquinone; DNP-INT, dinitrophenylether iodonitrothymol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; cytochrome *b*, cytochrome b_6; PMS, *N*-methylphenazonium methosulphate.

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membrane electron-transfer chain. Recently, Lavergne [1] proposed, on the basis of experiments performed with a mutant lacking of PS I, that a fifth electron carrier G is associated with the cytochrome *b/f* complex. According to Lavergne, this carrier is located close to the external face of the membrane and can exchange electrons with one of the two *b*-cytochromes, very likely cytochrome b_h. A transmembrane potential induces an electron transfer from G to cytochrome *b*, in modifying the equilibrium constant between these two carriers. The oxidized-minus-reduced spectrum of G presents a negative peak around 420 nm and no characteristic band in the green region. However, a complete spectrum of G was not obtained, due to the difficulty to subtract the numerous superimposed absorption changes (Q_A, electrochromic shift, cytochromes *b*, plastoquinone).

Most of the experiments described here were performed in a mutant lacking of PS II under anaerobic conditions, where the plastoquinone pool and the primary and secondary PS I donors (P-700, plastocyanin, cytochrome *f* and Rieske protein) are maintained in their reduced state. Illumination by a flash induces the transfer of a positive charge to the high-potential chain of the cytochrome *b/f* complex and the formation of a semiquinone at the plastoquinone site Z located close to the inner face of the membrane [2]. As shown in a previous paper [3], this semiquinone oxidizes either cytochrome b_l or cytochrome b_h, depending upon the experimental conditions. In this paper, we characterize the flash-induced difference spectra of cytochrome b_l, cytochrome b_h and G in reducing conditions. We then analyze the re-reduction of these carriers under various experimental conditions. The light-induced reduction of cytochrome b_h and G is also studied in oxidizing conditions in a mutant lacking of PS I. Finally, we determine the relative redox potentials of the carriers of the low-potential chain and discuss our results with respect to the possible mechanisms of electron transfer in the cytochrome *b/f* complex.

Materials and Methods

The experiments were performed using two double mutant strains, S52 and S56, of *Chlorella sorokiniana*, isolated by P. Bennoun. Both mutants have a small pigment antenna; in addition, S52 totally lacks of PS II while S56 lacks of PS I [4,5]. The algae were suspended in a 50 mM phosphate buffer, pH 6.5, 10% ficoll (w/w). Unless otherwise stated, 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 (algae were first bubbled with nitrogen). In the experiments of Figs. 9 and 10, the algae were treated with benzoquinone as follows: the algae were incubated for 10 min in the presence of 0.3 mM benzoquinone. The cells were then rapidly pelleted, washed once in a 50 mM tricine (pH 7.5), 50 mM KCl medium, centrifuged again, and finally resuspended in 50 mM phosphate buffer (pH 6.5) with 10% ficoll (w/w).

Spectrophotometric measurements were performed with an apparatus similar to that described in Ref. 6 and improved according to Ref. 7. Actinic excitation was provided by a xenon flash (3 μs duration at half-height), filtered through a Schott filter RG8.

In the α-band, the redox changes of cytochrome *b* were measured as the difference between the absorption at 564 nm and a baseline drawn between 545 nm and 575 nm. This procedure eliminates the contributions of cytochrome *f*, plastocyanin and G. Cytochrome *f* was measured at 553.5 nm from the same baseline and corrected for the cytochrome *b* contribution, which was estimated to 13% of the cytochrome *b* signal.

In the blue region of the spectrum cytochrome *b* concentration was estimated as the difference (433 nm–413 nm); at these wavelengths, the contribution of G is negligible. G was characterized by the difference (424 nm–444 nm); at these wavelengths, the contribution of the cytochrome *b* signal was estimated to

$$\Delta I/I_{424\text{ nm}-444\text{ nm}} = -0.08\,\Delta I/I_{433\text{ nm}-413\text{ nm}}$$

The concentration of cytochrome *b* was related to the concentration of cytochrome *f*, assuming two cytochrome *b* per cytochrome *f*. In the case of mutant S52, a full oxidation of cytochrome *f* was obtained by a 10 s strong continuous illumination of the algae dark-adapted in anaerobic

conditions in the presence of 0.03 mM DNP-INT, which is known to inhibit plastoquinol oxidation at site Z [8]. Using the absorption coefficients calculated by Rich et al. [9], we computed that, at the characteristic wavelengths mentioned above, the absorption changes due to the oxidation of one cytochrome *b* is 1.15 times the absorption changes due to the oxidation of one cytochrome *f*.

Results

Effect of membrane potential on cytochrome b oxidation

In Fig. 1, anaerobic dark-adapted algae were illuminated by a single over-saturating flash in the absence or in the presence of the ionophore dicyclohexyl-18-*crown*-6, which collapses the membrane potential [10]. As reported in Ref. 3, the oxidation of cytochrome *b* is biphasic in the absence of dicyclohexyl-18-*crown*-6; Fig. 1 (curves 1, A and B) shows that the slower phase of cytochrome *b* oxidation is synchronous with the decay of the membrane potential, monitored by the electrochromic absorption change (512 nm–475 nm). The addition of dicyclohexyl-18-*crown*-6 significantly increases the amount of cytochrome *b*

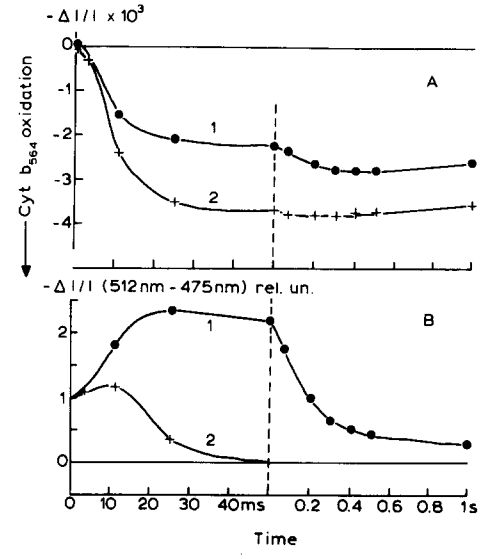


Fig. 1. Time-course of cytochrome *b*-564 oxidation (A) and of the field-indicating absorption change (512 nm–475 nm) (B) after saturating flashes 10 min apart. Mutant S52. Curves 1 (●): control. Curves 2 (+): 0.2 mM dicyclohexyl-18-*crown*-6. The amplitude of the field-indicating absorption change has been normalized to the fast phase (phase a).

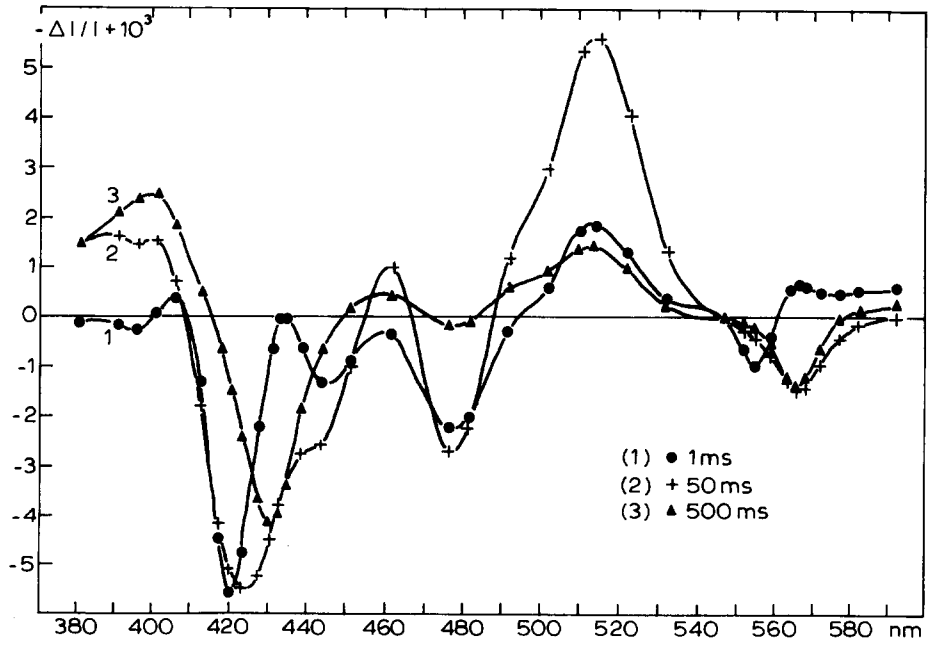


Fig. 2. Time-resolved spectra after saturating flashes 30 s apart. Mutant S52. Detecting times (1, ●) 1 ms; (2, +) 50 ms; (3, ▲) 500 ms.

oxidized by the flash and suppresses the slower phase of the kinetics (curve 2A).

Fig. 2 shows the spectra at various times following saturating flashes given 30 s apart. We previously proposed that the dark re-reduction of cytochrome b_h was completed in about 30 s, while more than 20 min was required to re-reduce cytochrome b_l [3]. Therefore, the 30 s dark interval between flashes is long enough to allow the reduction of most cytochrome b_h and short compared with the reduction time of cytochrome b_l, so that most of the centers were in the $b_h b_l^+$ state before each flash. Spectrum 1 (1 ms after each flash), mainly reflects the oxidation of cytochrome *f* and the electrochromic shift associated with the formation of the membrane potential due to the photochemical charge separation in PS I (phase a). Minor asborption changes are due to the oxidation of plastocyanin. 50 ms after the flash (spectrum 2), the membrane potential has reached its maximal value (phase b). The α-band of cytochrome *f* is no longer present while a signal due to cytochrome *b* oxidation appears at 564 nm; in the blue region of this spectrum, a large negative band peaking around 424 nm is observed. 500 ms after the flash (spectrum 3), most of the membrane potential is collapsed and the spectrum mainly reflects the oxidation of cytochrome *b* with negative peaks around 433 nm and 564 nm.

We thus observe in the presence of a membrane potential a signal peaking around 424 nm, which could be associated with the oxidation of G.

Spectrum of cytochrome b_h

In the experiment of Fig. 3, algae were submitted to repetitive saturating flashes, 18 s apart in the presence of dicyclohexyl-18-*crown*-6. Spectrum 1 shows the difference between the absorption measured 80 ms and 3 s after each flash. In this time range, we expect a large reduction of cytochrome b_h, but no significant re-reduction of cytocrome b_l. Nevertheless, spectrum 1 in the green region differs from the oxidized-minus-reduced spectrum of cytochrome b_h we measured in

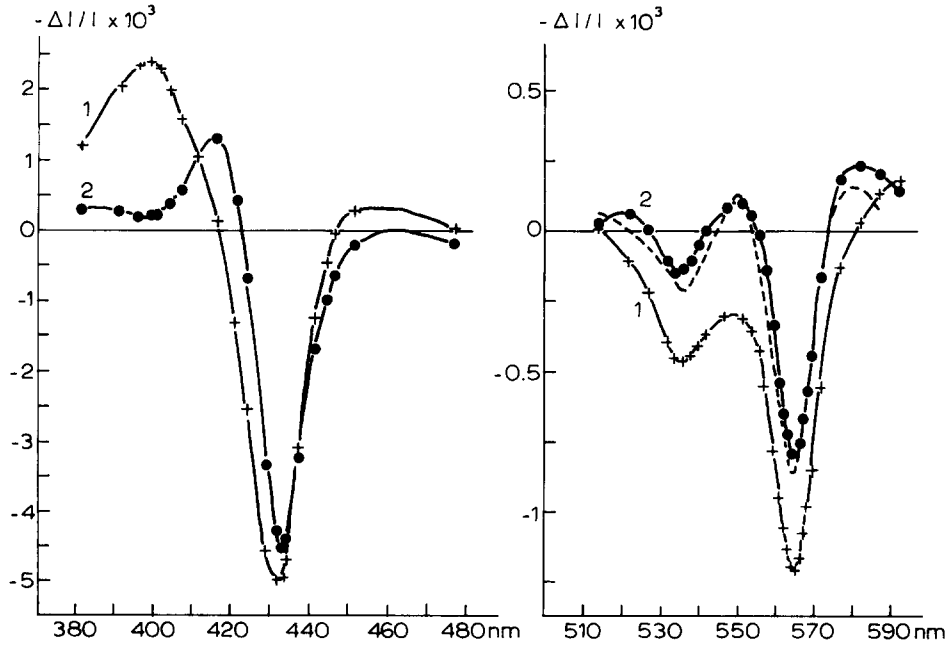


Fig. 3. Oxidized-minus-reduced spectrum of cytochrome b_h. Mutant S52. Spectra 1 (+): difference between the absorption measured 80 ms and 3 s after each flash of a series of saturating flashes 18 s apart. 0.3 mM dicyclohexyl-18-*crown*-6. Spectra 2 (●): spectrum of cytochrome b_h after subtraction of the contribution of G (see Discussion). The dashed spectrum (inverted for comparison) was obtained with isolated broken spinach chloroplasts, in the presence of 1 mM ferricyanide, 2 μM valinomycine and 50 mM KCl. It was measured 60 ms after a group of two flashes 1 s apart given to dark-adapted chloroplasts.

isolated spinach chloroplasts (dashed spectrum, conditions indicated in the legend): for instance, in isolated chloroplasts, the absorption around 550 nm is close to zero while it is negative in algae; also, the slope for wavelengths beyond 585 nm is negative in isolated chloroplasts, while it is positive in algae.

In the blue region, the Soret band around 433 nm is dissymmetrical and wider than that of a pure cytochrome *b* spectrum [11]. We conclude that spectrum 1 reflects the superimposition of two spectral changes, cytochrome b_h, and very likely, G.

Spectrum of G

In Fig. 4, spectrum 1 was measured 90 ms after each flash of a series of saturating flashes 5 s apart and is similar to spectrum 2 in Fig. 2. Spectral changes due to the formation of the membrane potential and to the oxidation of G and cytochrome b_h contribute to spectrum 1. Spectrum 2 was obtained with algae in the presence of a high concentration of PMS (400 μM) and 3 mM sodium dithionite. After dark adaptation under such conditions, all the electron carriers in the cytochrome *b/f* complex are in their reduced state. Upon illumination, a major fraction of the positive charges formed on the donor side of PS I is directly transferred to PMS; the remaining fraction induces cytochrome b_l oxidation. In this experiment (spectrum 2) the algae were illuminated with continuous light for 200 ms and then dark-adapted for 120 ms. These conditions were set in order to build up the same membrane potential and to oxidize the same amount of cytochrome *b* as in spectrum 1. On the other hand, the illumination should not induce the oxidation of G as most of the positive charges formed by PS I by-passes the cytochrome *b/f* complex and that the redox potential of cytochrome b_l is lower than those of cytochrome b_h and G. The oxidized-minus-reduced spectrum of G (spectrum 3) results from the difference between spectra 1 and 2. It is worthwhile to note that the spectrum of G is computed without any normalization of spectra 1 and 2, which excludes any error due to a non-linear response of

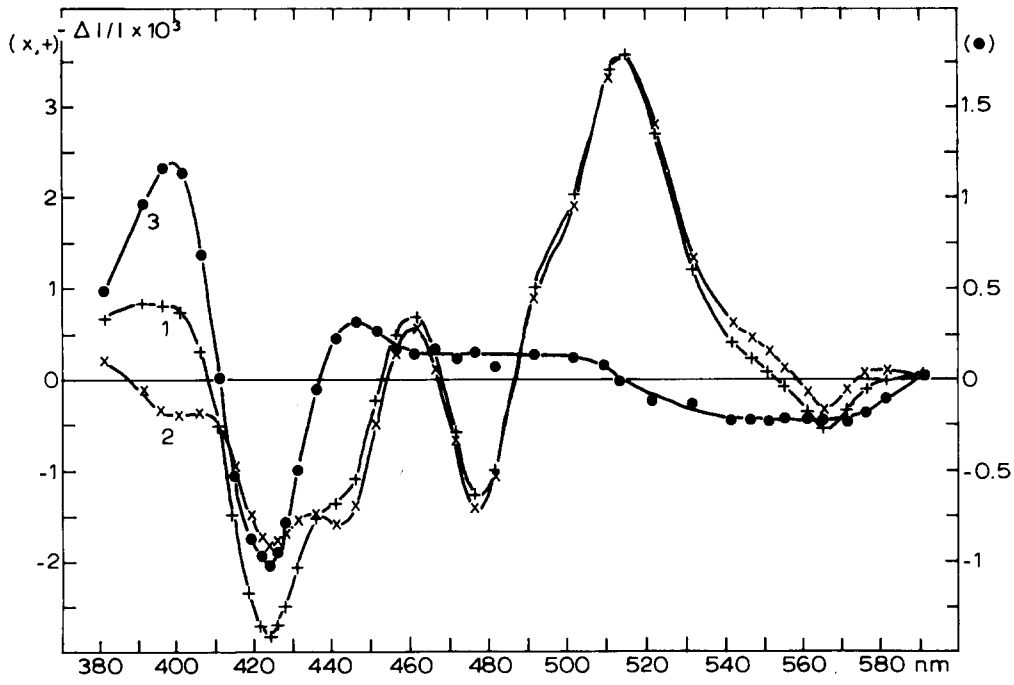


Fig. 4. Oxidized-minus-reduced spectrum of G. Mutant S52. Spectrum 1 (+): absorption changes measured 90 ms after a series of saturating flashes 5 s apart. Spectrum 2 (×): absorption changes measured 120 ms after a 200 ms-strong continuous illumiantion. 400 μM PMS; 3 mM sodium dithionite. Spectrum 3 (●): difference between spectra 1 and 2 (right ordinate scale expanded by a factor 2).

some of the electrochromic probes to the membrane potential. On the other hand, minor errors are introduced, since different *b*-cytochromes are oxidized in both experiments (cytochrome b_h in spectrum 1 and cytochrome b_l in spectrum 2). The G-spectrum (spectrum 3) displays a large negative band around 424 nm (very likely a double peak) and a broad and weak negative band in the green region. Both these features are characteristic of high-spin cytochromes of type *c'*, a class of soluble cytochromes identified in several types of photosynthetic bacteria (reviewed in Ref. 12). This cytochrome *c'* should be attached to the cytochrome *b/f* complex and located on the outer face of the membrane. In agreement with Lavergne [1], the equilibrium between G and cytochrome *b* – very likely cytochrome b_h – is modulated by the membrane potential.

Effect of CO

The various *c'* type cytochromes are known to bind CO, usually with a rather low affinity [12–14]. In Fig. 5, algae were submitted to a series of flashes 21 s apart in the absence or in the presence of CO. The flash energy was slightly under saturation in order to limit an eventual photodissociation of a G-CO complex. Fig. 5 shows that spectra 2A and 2B (in the presence of CO) are closer to a pure cytochrome b_h spectrum than spectra 1A and 1B (control). The amount of cytochrome b_h and G oxidized by the flash is smaller in the presence than in the absence of CO; as the experiment is performed in repetitive flash-illumination, we conclude that the amount of cytochrome b_h and G re-reduced during the 21 s interval between flashes is decreased by the addition of CO. Spectra 3 are the difference between spectra 1 and 2, after normalization to the same amount of cytochrome b_h. Spectra 3A and 3B are close to the spectrum of G (Fig. 4, spectrum 3).

Spectrum of cytochrome b_l

The anaerobic reduction of cytochrome b_l is a slow process achieved in 30–60 min [3]. In Fig. 6 is shown the difference between spectra obtained under the two following conditions: (a) algae were dark-adapted for 60 min, which induces a maximal level of cytochrome b_l reduction; (b) algae

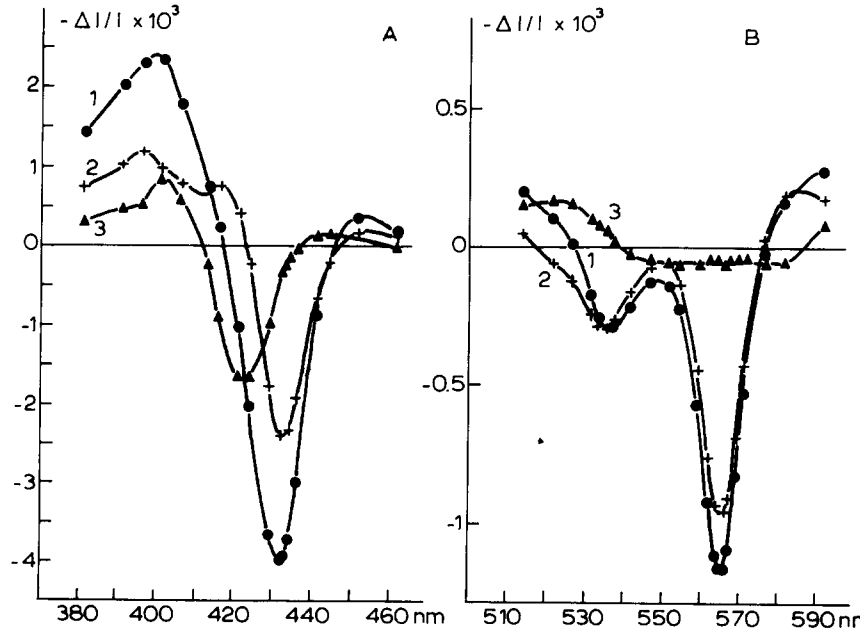


Fig. 5. Effect of CO on flash-induced absorption changes (time interval between flashes, 21 s; detecting time, 500 ms). Mutant S52. 0.3 mM dicyclohexyl-18-*crown*-6. Spectra 1 (●): control; spectra 2 (+): algae were bubbled with CO for 4 min. Spectra 3 (▲): difference between spectra 1 and 2, after normalization to the same amount of cytochrome *b*. Spectra in the blue (A) and the green (B) regions were performed with two different batches.

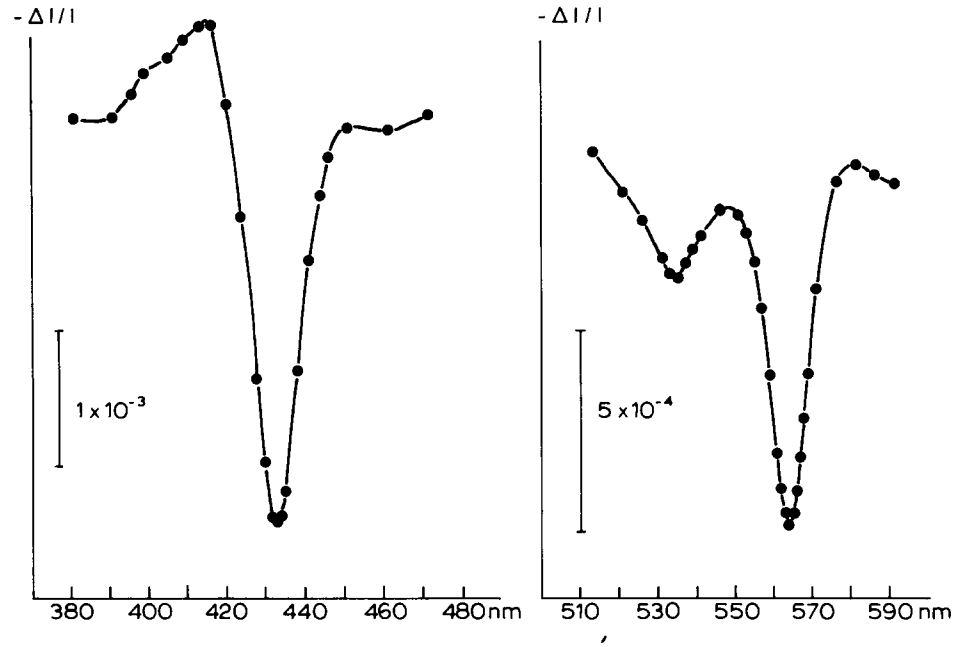


Fig. 6. Oxidized-minus-reduced spectrum of cytochrome b_l. Mutant S52. 0.3 mM dicyclohexyl-18-*crown*-6.

were preilluminated in weak light (1.2 photon per PS I center/minute) and then dark-adapted for 60 s. Under these conditions, we are sure that cytochrome b_h is fully reduced while cytochrome b_l is about half-oxidized. This spectrum presents a positive peak around 415 nm and negative peaks around 433 nm, 534 nm and 563.5 nm. The method does not permit an accurate determination of the isobestic points, because of a possible drift of the baseline during the time interval between the experiments in conditions a) and b).

Reduction of cytochromes b in mutant S52

Fig. 7 shows kinetics of anaerobic reduction of the *b*-cytochromes, using two batches of algae (curves 1 and 2) from different algal cultures. An absolute scale for the redox state of the cytochromes is used in this figure, calibrated as follows: in a first experiment, the absorption was measured in the 545 nm–575 nm range, in conditions that the two cytochromes *b* are fully reduced (1 h dark incubation in the presence of 3 mM sodium dithionite plus 10 μM phenazine methosulfate). In a second experiment, the absorption was measured in anaerobic conditions, after several minutes of a weak continuous illumination (1.2 photons per PS I center per min). The steady-state concentration of cytochrome *b* oxidized by the weak illumination is measured by the difference between spectra observed under these two experimental conditions. This concentration is normalized to the total concentration of cytochrome *f* (see Materials and Methods). The weak illumination induces the oxidation of 1.05 and 1.25 molecules of cytochrome *b* per cytochrome *b/f* complex for batches 1 and 2, respectively (dotted lines). The analysis of the α-band of the spectra (not shown) indicates that the weak illumination induced in both batches the total oxidation of cytochrome b_l, with an additional oxidation of cytochrome b_h (about 20%) for batch 2.

Fig. 7, part A shows the reduction of cytochrome *b* after illumination of the algae by a group of two saturating flashes superimposed on the weak illumination. These flashes induce an oxidation of cytochrome b_h only. No increase in cytochrome b_h oxidation is observed when the number of flashes is increased.

Fig. 7, part B shows the reduction of cytochrome *b* after switching off the continuous beam. After a 30–60 min dark period, a steady state is reached, which corresponds to 0.08 and 0.13 mole-

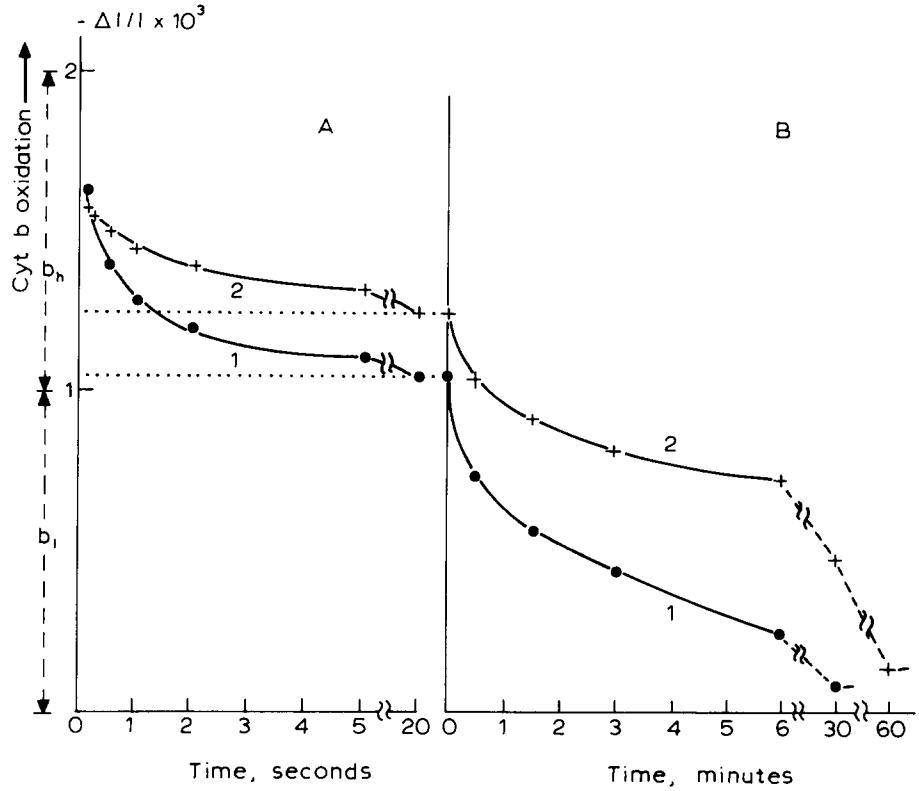


Fig. 7. Kinetics of reduction of cytochrome (cyt) *b* following various conditions of illumination. Mutant S52 with 0.3 mM dicyclohexyl-18-*crown*-6. Dotted lines: steady-state level of oxidation of cytochrome *b* under a weak continuous illumination (1.2 photon per PS I center/min). (A) Algae were preilluminated by a group of two oversaturating flashes superimposed on the weak continuous light. (B), Reduction of cytochrome *b* after switching off the weak light. Note the different time scales for A and B. Curves 1 (●) and 2 (+) correspond to two different algal cultures (in text, batches 1 and 2, respectively).

cules of oxidized cytochrome b_l for batches 1 and 2, respectively. The overall half-time for cytochrome b_l reduction (curves 1B and 2B) is about 200-times longer than that of cytochrome b_h reduction (curves 1A and 2A). The reduction rate of both cytochrome b_h and cytochrome b_l is slower for batch 2 than for batch 1.

Reduction of G

In Fig. 8, the anaerobic re-reduction of cytochrome b_h (curve 1) and G (curve 2) were measured in the Soret band using batch 1 under the conditions of Fig. 7, curve 1A. The reduction of G is faster than that of cytochrome b_h. However, this latter kinetics may be decomposed in a fast component (curve 1′) and a slower one (curve 1″). The fast one roughly follows the kinetics of G reduction ($t_{1/2} \approx 300$ ms). During the fast phase,

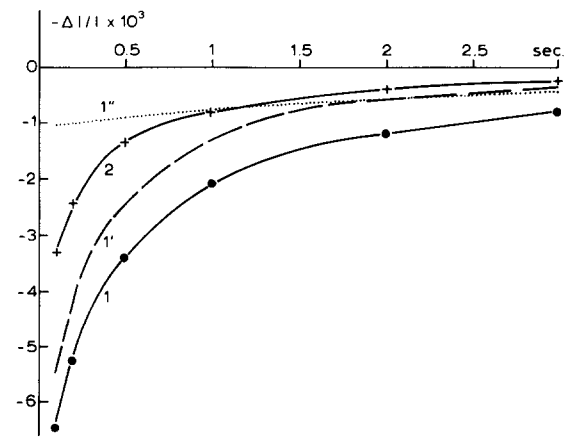


Fig. 8. Reduction kinetics of G and cytochrome b_h after a group of two flashes. Same conditions as in Fig. 7, curve 1A. G and cytochrome b_h were measured as discussed in Materials and Methods. Curve 1 (●): reduction of cytochrome b_h. Curve 2 (+): reduction of G. Curve 1 was analyzed as a sum of a fast (curve 1′) and a slow (curve 1″) component.

the ratio between the G and cytochrome b_h absorption changes is equal to about 0.6 (see curves 1′ and 2). Similar conclusions were drawn using batch 2 (data not shown), except that the relative amplitude of the slow component is larger.

Reduction of cytochrome b and G in mutant S56 lacking of PS I centers

The experiments in Figs. 9 and 10 were performed with benzoquinone-treated algae. This treatment makes the cell and the chloroplast envelopes permeable to ions [15,16], blocks ATPase activity [17] and the respiratory pathway [18]. In Fig. 9, algae were illuminated for 1.6 s with strong light. Spectra 1 and 2 measured at, respectively, 400 ms and 850 ms after the onset of illumination mainly reflect a reduction of cytochrome *b*, on which are superimposed absorption changes due to the membrane potential and to the reduction of PS II acceptor Q_A. Absorption changes in the 540 nm–550 nm range are mainly due to Q_A^-, which reaches its maximum concentration in about 400 ms. After subtraction of the C-550 signal, no significant signal can be ascribed to cytochrome *f* oxidation as already shown by Lavergne [1]. After 1.6 s of illumination (spectrum 3), the membrane potential has decreased by a factor of about 2 and an absorption band arises around 420 nm. In the green region, spectra 2 and 3 are similar, which shows that the concentration of reduced cytochrome *b* and Q_A^- does not significantly vary in this time range. The difference in the blue region is very likely due to the reduction of G associated with the decrease of the membrane potential. At 370 nm, the decrease in absorption observed during the course of illumination is mainly due to the reduction of the plastoquinone pool [19].

In Fig. 10 algae were illuminated for 3 s, in the presence of the ionophore dicyclohexyl-18-*crown*-6. During the course of illumination, the reduction of Q_A is achieved at a much shorter time than that of cytochrome *b* and G; consequently, Spectrum 1, which is the difference between the spectra measured after 3 s and 1 s illumination, mainly reflects the reduction of cytochrome *b* and G; the maximum in the blue region is around 428 nm, midway value between the peak of G (424 nm) and that of cytochrome *b* (433 nm); in the green region, the spectrum also reveals the reduction of cytochrome *b* superimposed on the reduction of G (negative slope between 580 nm and 590 nm). The ratio between the absorption changes which could

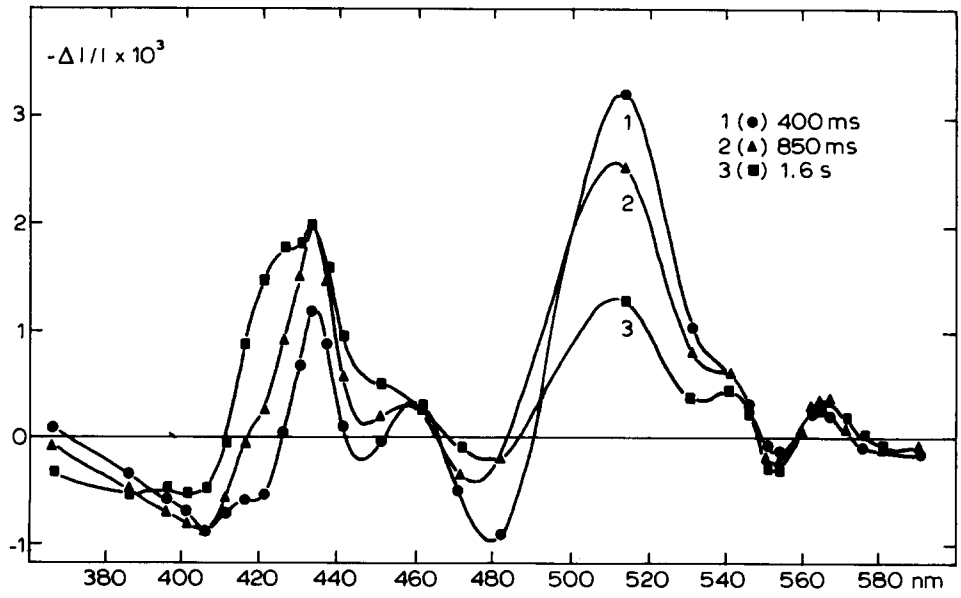


Fig. 9. Spectral changes induced by a strong continuous illumination of benzoquinone-treated mutant S56. Spectra 1 (●), 2 (▲) and 3 (■) are measured after 400 ms, 850 ms and 1.6 s of illumination, respectively.

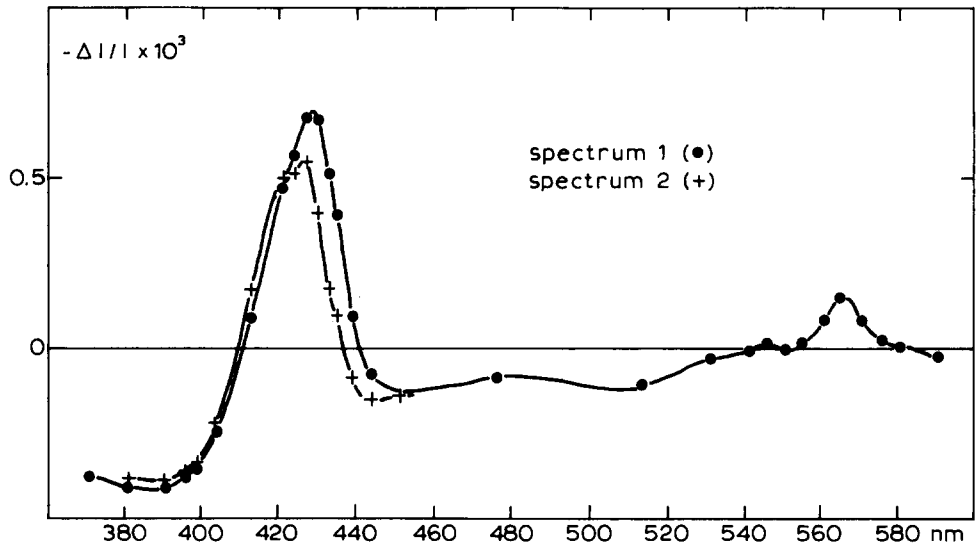


Fig. 10. Benzoquinone-treated mutant S56. 0.3 mM dicyclohexyl-18-*crown*-6. Spectrum 1 (●): difference between spectra measured after 3 s and 1 s of illumation; the spectrum at 1 s was multiplied by 1.15 in order to obtain the same amplitude of the C-550 signal as in the spectrum at 3 s. Spectrum 2 (+): reduced-minus-oxidized spectrum of G, after subtraction of the cytochrome b_h spectrum from spectrum 1.

be ascribed to the reduction of G and cytochrome b_h is 1.6. Spectrum 2 was obtained by subtracting the spectrum of cytochrome b_h (Fig. 3, spectrum 2), normalized to the same (433 nm–413 nm) signal. Spectrum 2 is close to the spectrum of G (Fig. 4, spectrum 3 and Fig. 5, spectrum 3A). This spectrum peaks around 424 nm, with a half-band width of 19 nm. At wavelengths shorter than 400 nm, the spectrum is distorted by the absorption changes due to plastoquinone reduction.

Discussion

As we already showed [3], two phases can be distinguished in the process of cytochrome *b* oxidation (Fig. 1, curve 1). The slower phase is synchronous with the decay of the membrane potential and disappears in the presence of dicyclohexyl-18-*crown*-6. These results can be interpreted assuming a membrane potential-dependent equilibrium between G and cytochrome b_h. In the presence of a membrane potential, a fraction of the positive charges appearing in the low-potential electron-transfer chain of cytochrome *b/f* complex is localized on G. A reverse transfer of positive charges from G to cytochrome b_h is associated with the decay of the membrane potential. It is interesting to note that the slow phase is only observed with high-energy flashes able to induce a large membrane potential (see Fig. 3 in Ref. 3).

On the basis of a theoretical analysis of the membrane potential-induced oxidation of G and reduction of cytochrome *b*, Lavergne [1] concluded that the redox potential of G was at the utmost 30 mV more positive than that of cytochrome *b*, which corresponds to an equilibrium constant of about 3. This implies that, even in the absence of a membrane potential, the oxidation of cytochrome b_h is always associated with the oxidation of G as in spectrum 1, Fig. 3. The pure spectrum of cytochrome b_h (spectrum 2, Fig. 3) was obtained by subtracting the spectrum of G (spectrum 3, Fig. 4) from spectrum 1, Fig. 3.

We observed that in the cytochrome b_h spectrum the ratio of the absorbances in the Soret band and the α-band can vary depending upon the cultures and the experimental conditions. From one culture to the other, changes in the chlorophyll content per cell can modify the flattening factor [20]. However, we sometimes observed a

decrease of about 25% of this ratio during the course of cytochrome b_h reduction. This phenomenon may suggest that cytochrome b_h can exist under several forms, which for instance, could differ by their degree of protonation.

Comparison of cytochrome b_h and cytochrome b_l spectra

After elimination of the contribution of G, the spectra of cytochrome b_h and cytochrome b_l appear very similar. In the blue region, both cytochromes peak around 433 nm, cytochrome b_h absorbing at a somewhat longer wavelength (about 0.3 nm). In the green region, the two *b*-cytochromes are more distinguishable: the position of the β-band (Figs. 3 and 6) is about 533 nm for cytochrome b_h and 534 nm for cytochrome b_l. As to the α-band (Fig. 11), cytochrome b_h peaks at 564 nm and cytochrome b_l at 563.4 nm; this difference of 0.6 nm is reproducible. One can also observe that the difference is less marked on the short-wavelength edge than on the long-wavelength edge of the band. The spectrum presented here is compatible with the spectra of cytochrome b_h and cytochrome b_l mesured at low temperature on isolated cytochrome *b/f* complexes [21]. We already reported a difference of about 0.6 nm between the maxima of cytochrome b_h and cytochrome b_l [3], but the spectrum of cytochrome b_h presented in this reference was not corrected for G.

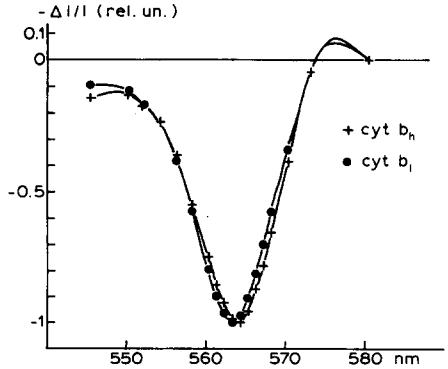


Fig. 11. Oxidized-minus-reduced spectra of cytochrome (cyt) b_h (+) and cytochrome b_l (●). Mutant S52. The two spectra were normalized to the same difference (564 nm–580 nm).

The absence of a significant contribution of G in the spectrum of cytochrome b_l can be interpreted in two ways: a) cytochrome b_h and cytochrome b_l are not in equilibrium; b) the equilibrium constant between these two components is larger than 10, which corresponds to a difference in their redox potentials of at least 60 mV.

Origin of the signal G

The signal G, first characterized in *Chlorella sorokiniana* [1], has been also observed in *Chlamydomonas reinhardtii* (Delosme, R., personal communication); we failed, however, to observe any signal which can be ascribed to G in isolated spinach chloroplasts (see Fig. 3). In the same way, we do not observe on this material any membrane potential induced change of the redox state of cytochrome b_h. We do not known whether G is absent in chloroplasts of higher plants, or inactivated or released in the medium during the chloroplasts preparation.

We can exclude that G could be identified with cytochromes of unknown function as cytochrome *b*-559LP or cytochrome *b*-560 [22]. The characteristic α-band of these cytochromes is not seen in the spectrum of G. It is also very unlikely that G could be a denatured high-spin form of cytochrome b_6 [23]. Actually, G is present at a rather constant concentration in living cells of *Chlorella* and *Chlamydomonas* and under various physiological conditions (aerobic or anaerobic). We have also considered the possibility that cytochrome b_h might be present under a low-spin and a high-spin form, in a membrane potential-dependent equilibrium. Our data and those of Lavergne [1] are not consistent with this hypothesis.

The spectrum of signal G (Fig. 4) is in reasonable agreement with those of *c'*-type cytochromes which have been isolated from different photosynthetic or non-photosynthetic bacteria [12]. Cytochromes *c'* difference spectra display a double peak in the blue region and a broad and weak band in the green region. Nevertheless, the oxidized-minus-reduced spectrum of cytochrome *c'* generally peak around 429 nm, i.e., at a longer wavelength than the spectrum of G (424 nm). The function of cytochromes *c'* is unknown. In photosynthetic bacteria, cytochrome *c'* is supposed to be located in the periplasmic space which is ho-

mologous to the inner space of the chloroplast thylakoids, while G is located on the opposite side of the membrane, i.e., on the stroma side.

In isolated cytochrome *b/f* complexes from *Chlamydomonas reinhardtii* [24], we did not observe any contribution of G in the oxidized-minus-reduced spectra (in collaboration with Wollman, F.A. and Lemaire, C., unpublished results). This result suggests that *G*, as cytochrome c' , is a soluble protein weakly bound to the cytochrome *b/f* complex and lost during the preparation of the isolated complexes.

Gibson and Kamen [13] showed that cytochrome c' is able to bind CO through a rather complex mechanism. The cytochrome c'–CO dissociation constant k_d is highly variable (10^{-3} M $> k_d >$ 10^{-6} M) depending upon the bacterial species [14]. We observed that in the presence of a CO-saturated solution (about 1 mM), the oxidation of G is not fully blocked, which implies that the value of the CO-dissociation constant is between $1 \cdot 10^{-4}$ M and $5 \cdot 10^{-4}$ M. The redox potentials of cytochromes c' (around 0 to 150 mV) are pH-dependent in a complex manner. Most of them have a redox potential close to 0 mV, which make them good candidates for an electron carrier in close equilibrium with cytochrome b_h ($E_m \approx 0$ mV [21]).

Mechanisms for cytochrome b reduction in anaerobic conditions

The weak continuous illumination of the algae in anaerobic conditions induces a steady state in which cytochrome b_l is fully oxidized. When two saturating flashes are superimposed on the weak illumination, a large excess of positive charges per cytochrome *b/f* complex is available among PS I donors. The model presented in Ref. 3 predicts that under these conditions about 0.5 molecules of cytochrome b_h and 1 molecule of cytochrome b_l should be oxidized. These values are close to the experimentally observed ones. Comparison of parts A and B (Fig. 7) shows that the overall half-time of reduction of cytochrome b_h is about 200-times shorter than that of cytochrome b_l. If one assumes that: (a) the electrons from an unknown source are transferred first to cytochrome b_h and then to cytochrome b_l; (b) the rate of electron transfer to the cytochromes *b* chain is proportional to the concentration of the oxidized form of cytochrome b_h (first-order process); and (c) cytochrome b_h and cytochrome b_l are in equilibrium, then the equilibrium constant between cytochrome b_h and cytochrome b_l should be equal to the ratio of the half-times of reduction of cytochrome b_h and cytochrome b_l. To an equilibrium constant of 200 corresponds a redox potential gap of about 140 mV. Our interpretation is strengthened by the fact that, from one batch to the other, the ratio between the half-times of reduction of cytochrome b_h and b_l remains approximately constant, while the absolute values of these half-times are variable. Our data agree with the titration performed by Hurt and Hauska [21] in isolated cytochrome *b/f* complexes from spinach, who found a difference of potential of about 140 mV between cytochrome b_h and cytochrome b_l.

The presence of the two phases observed – specially in batch 2 – in the kinetics of cytochrome *b* reduction suggests that there are two populations of cytochrome *b/f* complexes which differ by the rate of cytochrome *b* reduction. The reduction of G follows the fast reduction phase of cytochrome b_h (Fig. 8), which suggests that G is associated with only one of the populations of the cytochrome *b/f* complexes. We conclude that, very likely, G takes part in a new electron-transfer chain, which is able to transfer electrons from stromal reductants to the *b*-cytochromes. This chain is specifically inhibited by CO which actually slows down the reduction of the low-potential chain components.

The rate constant for the reduction of G largely varies from one culture to the other (half-times from 300 ms to a few seconds). This indicates that other parameters than the presence of G control the rate of electron transfer via the chain which feeds cytochrome b_h in electrons; these parameters could be either the concentration of a soluble reductant or an enzyme which catalyzes electron transfer between this reductant and G.

Several mechanisms can be proposed for the slower phase of reduction of the *b*-cytochromes among which: (a) the source of electrons could be PQH_2 via a slow reaction occurring at plastoquinone site C; (b) G in solution is in equilibrium with G bound to the cytochrome *b/f* complexes; cytochrome *b* reduction would be then

controlled by the rate of exchange of G with the different cytochrome *b/f* complexes.

Mechanisms of reduction of the b-cytochromes in mutants lacking of PS I

Illumination of benzoquinone-treated algae induces a partial reduction of the components of the low-potential electron-transfer chain in the cytochrome *b/f* complex. In the presence of a large membrane potential (Fig. 9, curve 1), only cytochrome b_h is reduced, while in the presence of dicyclohexyl-18-*crown*-6, a parallel reduction of cytochrome b_h and of G is observed (Fig. 10). We estimate that a strong continuous illumination can induce the reduction of no more than 0.25 molecule of cytochrome b_h per cytochrome *b/f* complex.

The mechanism through which PS II-turnover induces the reduction of a fraction of cytochrome b_h and G is unknown. The involvement of plastoquinol at site Z is unlikely as we do not observe spectral changes which could be ascribed to the reduction or the oxidation of cytochrome *f*. Lavergne [1] suggested that a low-potential PS II auxiliary acceptor (Q_L [25], Q_2 [26]) could directly transfer electrons to cytochrome *b*. We observe that the reduction of cytochrome b_h and G approximately follows the reduction of the plastoquinone pool (data not shown). This suggests that the electrons are transferred from PQH_2 to cytochrome b_h via the plastoquinone site C. As proposed above, the same mechanism could be involved in the slow reduction of cytochrome *b* observed under anaerobic conditions in the mutant S52 lacking of PS II.

Equilibrium constant for the electron transfer between cytochrome b_h and G

The comparison of the light-induced partial oxidation of cytochrome b_h and G in mutant S52 and of their light-induced partial reduction in mutant S56 should permit an approximate determination of the equilibrium constant and the relative extinction coefficients for these electron carriers. For this estimation, cytochrome b_h and G will be characterized by the absorption changes (433 nm–413 nm) and (424 nm–444 nm), respectively.

We assume that G and the cytochrome *b/f* complex form a stable association and that no electron exchange occurs between different complexes.

In Fig. 8 (mutant S52), for the fraction of the cytochrome *b/f* complexes associated with G, the following sequence of reactions occurs:

$$\text{G} - \text{cytochrome } b_h$$

$$\xrightarrow{\text{light}} \text{G} - \text{cytochrome } b_h^+ \overset{K}{\rightleftarrows} \text{G}^+ - \text{cytochrome } b_h$$

The ratio of the absorption changes due to the oxidation of G and cytochrome b_h is about 0.6. Then, if ϵ_G and ϵ_b are the absorption coefficients for G and cytochrome b_h, respectively,

$$0.6 = \frac{\epsilon_G}{\epsilon_b} \frac{[\text{G}^+ - \text{cytochrome } b_h]}{[\text{G} - \text{cytochrome } b_h^+]} = \frac{\epsilon_G}{\epsilon_b} K \qquad (1)$$

In mutant S56 (Fig. 10), illumination of benzoquinone-treated algae in the presence of dicyclohexyl-18-*crown*-6 induces the reduction of G and cytochrome b_h:

$$\text{G}^+ - \text{cytochrome } b_h^+$$

$$\xrightarrow{\text{light}} \text{G}^+ - \text{cytochrome } b_h \overset{K}{\rightleftarrows} \text{G} - \text{cytochrome } b_h^+$$

The ratio of the signals due to the reduction of G and cytochrome b_h is 1.6. Then,

$$1.6 = \frac{\epsilon_G}{\epsilon_b} \frac{[\text{G} - \text{cytochrome } b_h^+]}{[\text{G}^+ - \text{cytochrome } b_h]} = \frac{\epsilon_G}{\epsilon_b} \frac{1}{K} \qquad (2)$$

From Eqns. 1 and 2, we deduce $\epsilon_G \approx \epsilon_b$ and $K \approx 0.6$, which corresponds to a midpoint potential for G of 10–20 mV more positive than that of cytochrome b_h.

Our estimation of the difference between the midpoint potentials of cytochrome b_h and G is close to that of Lavergne ($\approx$ 30 mV [1]) based on the analysis of the membrane-potential-induced electron transfer between these components. The small discrepancy could be due to an overestimation by Lavergne of the fraction of cytochrome b_h, which is reduced in the presence of membrane potential. On the basis of these measurements and the titrations performed by Hurt and Hauska [21] in isolated cytochrome *b/f* complexes from spinach, we proposed the following values for the

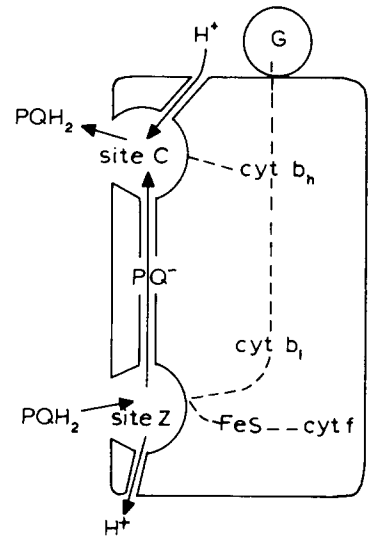
midpoint potentials of the carriers of the low-potential electron-transfer chain: G, $E_m \approx +10$ mV, cytochrome $b_h \approx 0$ mV and cytochrome $b_l \approx -140$ mV.

Properties of site C

Illumination of benzoquinone-treated mutant S56 induces the reduction of about 95% of the plastoquinone pool and of about 20% of cytochrome b_h. Reduction of cytochrome b_h very likely involves electron-transfer reactions occurring at site C between the couples PQ^-/PQH_2 and cytochrome b_h^+/cytochrome b_h. At the end of the illumination period, when a steady state is reached, these couples should be in equilibrium and their potentials close to 30 mV. In the same conditions, about 95% of the plastoquinone pool is reduced, which implies that the potential of the PQ/PQH_2 couple is about 20 mV. As the sum of the redox potentials of the two couples PQ/PQ^- and PQ^-/PQH_2 is equal to the potential of the PQ/PQH_2 couple, we conclude that the potentials of the two couples involving semiquinone are about equal. Therefore, the affinity of PQ^- for site C should be much higher ($\times 10^4$) than that of PQ and PQH_2. In this respect, the properties of site C appear very similar to those of the PS II acceptor site Q_B.

This conclusion is in contradiction with the interpretation we previously proposed [27] of experiments performed with isolated spinach chloroplasts in the presence of ferricyanide [7]: a group of two flashes given to dark adapted chloroplasts produces one plastoquinol molecule which induces the reduction of about 0.75 molecule of cytochrome b_h per cytochrome b/f complex. In spite of the large excess of plastoquinone (more than 95% of the pool oxidized), the reoxidation of cytochrome b_h is slow ($t_{1/2} \approx 1$ s) and probably mediated by ferricyanide. According to the conclusions of the preceding paragraph, we expect that the potential of the PQ/PQ^- couple should be around +200 mV, i.e., a value far more positive than that of the cytochrome b_h^+/cytochrome b_h couple (about −30 mV). Then, electron transfer between cytochrome b_h and PQ at site C appears kinetically limited.

Most of our results can be interpreted by a model (Scheme I), resembling the one of Wikström and Saraste [28] in which a charged semiquinone moves from site Z to site C through a specialized channel ('Q-pocket'). The higher affinity of semiquinone for site C than for site Z provides the driving force required to transfer PQ^- through the 'Q-pocket'. In this model, the classical Q-cycle mechanism would not be involved in the fast electron-transfer reactions occurring in the cytochrome b/f complex.



Scheme I.

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