Biochimica et Biophysica Acta, 1102 (1992) 53–61

BBABIO 43690

Electron transfer between Photosystem II and the cytochrome *b/f* complex: mechanistic and structural implications

Pierre Joliot and Anne Joliot

Institut de Biologie Physico-Chimique, Paris (France)

(Received 30 January 1992)

Key words: Electron transfer; Plastoquinone; Cytochrome *b/f*; Supercomplex; Q-cycle; Restricted diffusion

Electron transfer between Photosystem II (PSII) centers, cytochrome *b/f* complexes and Photosystem I (PSI) centers have been studied in isolated spinach chloroplasts in the presence of ferricyanide. The analysis of the reduction of cytochrome b_h under saturating illumination shows that about 2/3 of cytochrome *b/f* complexes are rapidly accessible to PSII-formed plastoquinol (< 10 ms), while for the remaining fraction, cytochrome b_h is reduced in the 100 ms range. These results are interpreted according to a model proposed by Joliot et al. (Joliot, P., Lavergne, J. and Béal, D. (1992) Biochim. Biophys. Acta 1101, 1–12), in which the diffusion of plastoquinone is restricted to domains, including an average of 4 PSII centers. Cytochrome *b/f* complexes included in the grana regions are involved in the linear electron flow from PSII to PSI; long-range electron transfer reactions are exclusively mediated by plastocyanin. Under flash excitation, the rate of electron transfer from PSII to PSI is limited neither by the diffusion nor by the binding of plastoquinol to cytochrome *b/f* complexes, but by electron transfer processes occurring within the cytochrome *b/f* complexes. The rate of these limiting processes is very likely controlled by the redox state of the high potential chain (cytochrome *f* and Rieske protein). Electron transfer through the cytochrome *b/f* complexes is discussed in terms of a Q-cycle or a semiquinone cycle mechanism.

Introduction

Electron transfer between PSII and PSI centers involves two mobile carriers. The liposoluble plastoquinone PQ mediates the electron transfer between PSII and cytochrome *b/f* complex and the hydrosoluble plastocyanin mediates the electron transfer between cytochrome *b/f* complex and PSI. PSII and PSI centers segregate in different regions of the membrane, appressed and non-appressed respectively, while the cytochrome *b/f* complex is distributed in both regions of the membrane (reviewed in Ref. 1). A crucial problem is to determine if one or both soluble carriers are involved in long-range electron transfer reactions. Kinetic and thermodynamic analyses of electron transfer from PSII to the PQ pool suggest that diffusion of plastoquinone is restricted to domains of small size, including an average of four PSII centers. These domains would be highly heterogeneous in terms of their absolute size and of the [PQ]/[PSII] stoichiometry [2–5]. A restricted diffusion of plastoquinone implies that only the cytochrome *b/f* complexes included in the grana regions are involved in the linear electron flow from water to NADPH and that long-range electron transfer is exclusively mediated by plastocyanin. We previously analysed electron transfer reactions from PSII to PSI in isolated chloroplasts in the presence of ferricyanide, i.e., in conditions where the PQ pool and both cytochrome b_h and cytochrome b_l are fully oxidized in dark-adapted material [6,7]. Excitation by a series of saturating flashes induces the reduction of less than half of the total cytochrome *b* content, despite the large excess of photochemically produced PQH_2. On the other hand, reoxidation of cytochrome *b* is a slow process ($t_{1/2} \approx 1$ s), which suggests that the Q-cycle is not efficiently operating under these conditions.

In this paper, we reinvestigate the process of electron transfer between PSII, cytochrome *b/f* and PSI complexes under flash and continuous illumination and

Correspondence to: P. Joliot, Institut de Biologie Physico-Chimique, 13, rue Pierre-et-Marie Curie, 75005 Paris, France.

Abbreviations: P700, Photosystem I primary donor; PQ, plastoquinone; PQH_2, plastoquinol; Q_Z and Q_C-sites, plastoquinone binding sites in the cytochrome *b/f* complex, close to the inner and the outer face of the membrane, respectively; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; NADPH, reduced nicotinamide diphosphate.

discuss the functional and structural implications of these experiments.

Materials and Methods

Market spinach leaves were ground in a medium containing 0.4 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 50 mM Hepes (pH 7.5), 2 mM sodium ascorbate and 2 g/l bovine serum albumin. The homogenate was filtered and centrifugated ($2000 \times g$, 10 min). The pellet was resuspended in the same medium and kept frozen in the presence of 5% dimethyl sulfoxide. The experiments were performed at room temperature, in a medium containing 50 mM phosphate buffer (pH 6.5), 5 mM $MgCl_2$, 1 μM gramicidin D, 0.1 mM methyl viologen. All the experiments but that of Fig. 6 were performed in the presence of 0.3 mM ferricyanide.

Spectrophotometric measurements were performed with an apparatus similar to that described in Ref. 8 and improved according to Ref. 6. Actinic flash excitation was provided by a xenon flash (3 μs at half-height), filtered through a Schott RG665 filter for cytochrome *f* and cytochrome *b* measurements, or a Corning 4-56 filter for P700 measurement. For these experiments, the chlorophyll concentration was 30 μg/ml. The light-detecting diodes were protected from scattered actinic illumination by Corning 4-56 filters for cytochrome *b* detection and by Kodak Wratten 96 + Wratten 16 filters for P700 measurement. Continuous actinic illumination was provided by two arrays of nine red light-emitting diodes (Toshiba TLRA-180x, peak wavelength around 660 nm) which illuminate opposite faces of the cuvette. To keep the continuous actinic illumination homogeneous, experiments were performed at a chlorophyll concentration of 15 μg/ml. At maximum operating power (5 mW/diode), the intensity corresponded to 0.5 to 1 absorbed photons per PSII center per ms.

The redox changes of cytochrome *b* are measured in the α-band, as the difference between the absorption at 563 nm and a baseline drawn between 545 nm and 573 nm. This procedure essentially eliminates the contribution of cytochrome *f*, P700 and plastocyanin. Cytochrome *f* was measured at 553 nm from the same baseline, and corrected for cytochrome *b* contribution, by subtracting 8% of the cytochrome *b* signal. Taking into account the absorption coefficients from Rich et al. [9], we computed that at the characteristic wavelengths mentioned above, the absorption coefficients for cytochrome *b* and cytochrome *f* were 21.5 and 17.4 mM^{-1}/cm, respectively. P700 was measured as the difference between the absorption at 700 nm and 729 nm. The absorption coefficient in digitonin PSI particules is 66 mM^{-1}/cm [10]. Taking into account a small flattening effect at 700 nm in chloroplasts, we estimate the absorption coefficient to be 62 mM^{-1}/cm.

The relative concentrations of PSI and PSII centers were measured by the amplitude of the electrochromic shift induced by a laser flash either when both PSII and PSI are active, or when PSII is blocked by addition of 2 mM hydroxylamine and 20 μM DCMU. The concentration of active PSII centers (those which include an active Q_B site) is ~ 0.90 of the concentration of PSI centers.

The relative amounts of photooxidizable P700 and cytochrome *f* were measured in the presence of 20 μM DCMU, 1 mM hydroxylamine and 6 mM sodium ascorbate. Chloroplasts were submitted to a saturating illumination for 100 ms, which fully oxidized P700 and the secondary PSI donors. The ratio [cytochrome *f*]/[P700] was thus estimated at 0.7.

The amount of chemically reducible cytochrome *b* (cytochrome b_h + cytochrome b_l) was measured as the difference between the chloroplasts absorption in the presence of about 10 mM sodium ascorbate and in the presence of about 10 mM sodium dithionite + 1 μM phenazine methosulfate. From the redox potentials of the different cytochromes, one expects that cytochrome *f* and high-potential cytochrome *b*-559 to be fully reduced in the presence of sodium ascorbate, while all of the *b* cytochromes, including cytochrome b_h, cytochrome b_l and low potential cytochrome *b*-560 are reduced in the presence of Na dithionite. Note that cytochrome *b*-560 is not associated with PSII [11] and thus differs from cytochrome *b*-559. On the basis of redox titrations, Kramer [12] characterized the spectra of cytochromes b_h, b_l and *b*-560, which peak at 563 nm, 565 nm and 560 nm, respectively. Their relative amounts were estimated as 1/1/0.6, respectively. The difference spectrum (dithionite minus ascorbate) peaks at 563 nm, which suggests that cytochrome *b*-560 is contributing to the difference spectrum, i.e., that a fraction of it is still oxidized in the presence of ascorbate. The amplitude of the differential signal is $\Delta I/I = 6.6 \cdot 10^{-3}$ for 30 μg/ml chlorophyll. On the basis of Kramer spectra [12] and assuming that 10 to 15% of the signal at 563 nm is due to cytochrome *b*-560, we estimated the concentration of the chemically reducible cytochrome *b* (cytochrome b_h + cytochrome b_l) to be 1.4 ± 0.1 of P700 concentration, in good agreement with our above estimate for cytochrome *f*.

We checked that ferricyanide or methyl viologen have no inhibitory effect on the rate of electron transfer reactions occurring at Q_Z or Q_C-sites. Actually, after a weak far-red illumination, which fully oxidized the secondary PSI donors, the same kinetics of flash-induced reduction or oxidation of cytochrome b_h were observed in the presence or absence of ferricyanide and methyl viologen.

After more than 1 min-dark incubation in the presence of 0.3 mM ferricyanide, ~ 30% of P700 is in the oxidized state, which corresponds to a redox potential

of 450 to 460 mV. After a light-induced oxidation of P700 in the presence of DCMU, i.e., when no PQH2 is formed by PSII, the re-reduction of P700 occurs with a half-time of about 10 s.

In the experiments reported here, the concentrations of the electron carriers were expressed relative to the P700 concentration.

Results

In Fig. 1, the reduction of cytochrome *b* was measured in chloroplasts in the presence of 0.3 mM ferricyanide after a series of one to five saturating flashes, given 12 ms apart (curves 1 to 5). Whatever the flash number, the absorption change peaked at 563 nm, which corresponds to the absorption maximum of cytochrome b_h in chloroplasts. Only a small amount of cytochrome b_h is reduced after the first actinic flash while about ten times more is reduced after the second one. Therefore, PQH_2 involved in the cytochrome b_h reduction arises from the PSII two-electron gate process. The significant reduction of cytochrome b_h observed after a single non-saturating flash (i.e., not inducing double hits), suggests that a fraction of secondary PSII acceptors is in the Q_B^- state in dark-adapted material, despite the strong oxidizing conditions. The kinetics of cytochrome b_h reduction measured after the last flash of each series is close to an exponential function and the half-time is independent of the flash number (approx. 12 ms). Therefore, the rate constant for cytochrome b_h reduction does not depend upon PQH_2 or oxidized cytochrome b_h concentrations, a characteristic behavior for a first-order reaction. The half-time for the reoxidation following

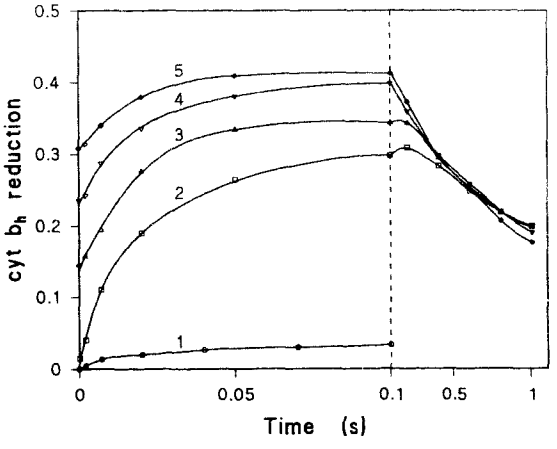

Fig. 1. Reduction of cytochrome b_h in chloroplasts submitted to a series of saturating flashes given 12 ms apart. Curves 1 to 5 correspond to a pre-illumination by one to five flashes, respectively. The last of a group of *n* flashes is given at time zero, and the corresponding ordinate is measured at time 12 ms on curve $(n-1)$.

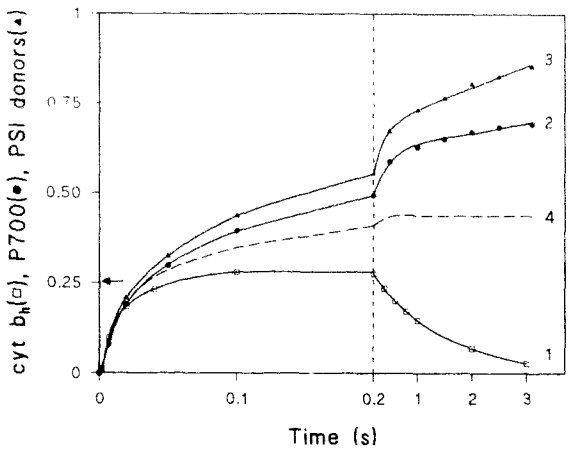

Fig. 2. Reduction of cytochrome b_h, P700 and secondary PSI donors induced by the second of a series of two saturating flashes given 200 ms apart. 1, cytochrome b_h; P700; 3, P700 + secondary PSI donors (plastocyanin, cytochrome *f* and Rieske protein); 4, cytochrome b_h corrected for cytochrome b_h oxidation (see Discussion). Arrow, amplitude of the fast phase for curves 1–4.

the second actinic flash is about 1.5 s and slightly decreases when the flash number increases. This slow reoxidation phase shows that cytochrome b_h cannot be rapidly reoxidized by PQ at site Q_C (as already shown in Refs. 6 and 7).

Fig. 2 shows the time-course of cytochrome b_h reduction (curve 1) and P700 reduction (curve 2) after the second of a group of two saturating flashes spaced 200 ms apart.

During the process of PQH_2 oxidation, the electrons primarily result in re-reduction of P700, while a small fraction of the electrons is stored in the PSI secondary donors (FeS center, cytochrome *f* and plastocyanin). We measured the amount of these reduced secondary donors at various times after the second flash by giving a third saturating flash. This third flash induces a full oxidation of P700, followed by a fast re-reduction phase, completed in about 2 to 3 ms, due to the transfer of electrons initially present in the secondary donors to P700. We checked that, in the conditions of our experiment, most of the electrons stored in the secondary donors prior to the third flash are transferred to P700 during this ms reduction phase. Thus, the amplitude of the ms phase following the third flash gives a measurement of the amount of reduced secondary donors present prior to this flash. Curve 3 shows the time-course of electron transfer to P700 and the PSI secondary donors, estimated in this manner.

Curves 1 and 2 are superimposed during the first 20 ms after the second flash, showing that during this phase, about the same number of electrons is transferred to P700 and to cytochrome b_h. At longer times

after the second flash, the amounts of reduced P700 (curve 2) or reduced P700 and secondary donors (curve 3) become much larger than reduced cytochrome b_h. Curve 3 displays three phases: a first phase ($t_{1/2} \sim 12$ ms) corresponds to the concerted reduction of cytochrome b_h and of the high potential chain; a second phase ($t_{1/2} \sim 150$ ms) is followed by a much slower one, not yet completed in 3 s. The amount of electrons stored in P700, secondary donors and cytochrome b_h 3 s after the second flash is ~ 0.9, which corresponds to the oxidation of ~ 0.45 PQH_2 molecules, in large excess of the amount of reduced cytochrome b_h ($\sim$ 0.27).

In Fig. 3, chloroplasts were illuminated by saturating flashes spaced 75 s apart in order to reach a steady-state regime. Due to the reoxidation of Q_B^- during this long dark period, the amount of PQH_2 produced is at least 4-times lower than in the conditions of Fig. 2 (flashes spaced 200 ms apart). Kinetics of cytochrome b_h reduction (curve 1) is clearly biphasic with a fast phase similar to that of curve 1, Fig. 2 and a slower phase which represents about 0.20 of the total amplitude. Surprisingly enough, despite the limiting amount of PQH_2 generated by the flashes, the slow phase in the reduction of cytochrome b_h shows that a fraction of PQH_2 has no rapid access to the cytochrome b/f complex. Cytochrome b_h reoxidation is about 2-times slower than in the experiment of Fig. 2. Curve 2 shows the kinetics of P700 reduction associated with PQH_2 reoxidation. Under these conditions, the rate of P700 reduction measured in the absence of PQH_2 (see Materials and Methods) is not negligible compared to the rate of P700 reduction associated with PQH_2 oxidation. Thus, curve 2 was computed by subtracting the

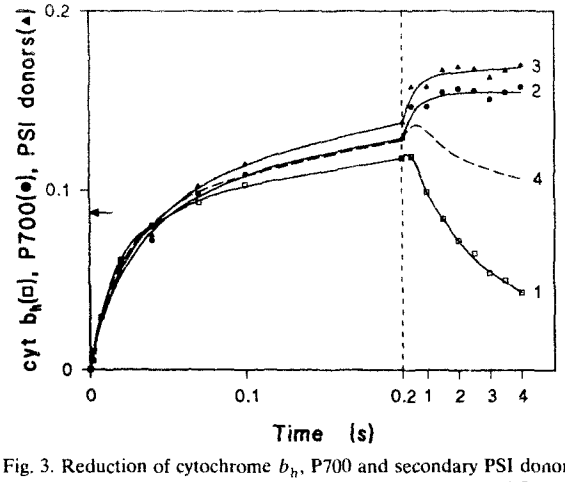

Fig. 3. Reduction of cytochrome b_h, P700 and secondary PSI donors induced by flashes given 75 s apart. 1, cytochrome b_h; 2, P700; 3, P700 + secondary PSI donors (plastocyanin, cytochrome f and Rieske protein); 4, cytochrome b_h corrected for cytochrome b_h oxidation (see Discussion). Arrow, amplitude of the fast phase for curves 1–4.

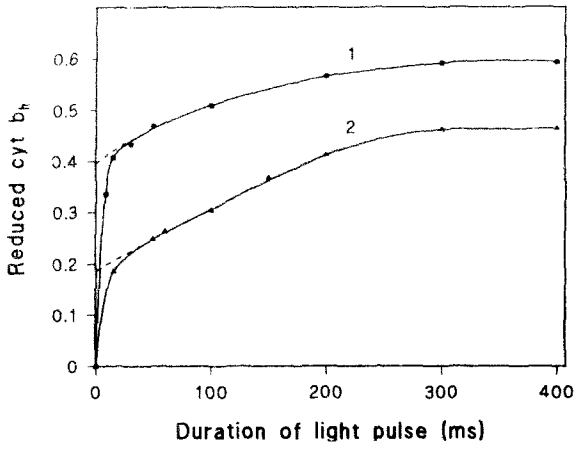

Fig. 4. Reduction of cytochrome b_h measured after saturating light pulses of various duration. For each pulse duration, the maximum amount of reduced cytochrome b_h is measured during the subsequent dark period. 1, Control; 2, 0.1 μM DCMU.

time-course of P700 reduction measured in the presence of a saturating concentration of DCMU from the time-course of P700 reduction measured in the absence of DCMU. Curve 3 shows the reduction kinetics of P700 and secondary PSI donors as a function of the dark-time following the flash, using the same procedure as in Fig. 2, curve 3. Curves 1, 2 and 3 are superimposed during the first 20 ms following the flash, as already observed in the experiment of Fig. 2.

In Fig. 4, chloroplasts were illuminated by pulses of saturating light of various duration and the maximum level of reduced cytochrome b was plotted against the duration of the pulse. For each pulse, cytochrome b was measured during the subsequent dark period, after completion of all the reactions leading to cytochrome b reduction (diffusion and binding of PQH_2; electron transfer within the cytochrome b/f complex). Thus, we measured the fraction of cytochrome b/f complexes able to interact with PQH_2 for a pulse of given duration. (Kinetics shown in Fig. 4 must not be compared to the flash-induced kinetics of Figs. 1, 2 and 6.) The absorption spectrum of reduced cytochrome b was measured after a short (10 ms) or long (300 ms) pulse. Both spectra peak at 563 nm (not shown), which corresponds to the absorption peak of cytochrome b_h [12]. In Fig. 4, curve 1 is markedly biphasic. The amplitude of the fast phase is about 0.40, which corresponds to $\sim 57\%$ of chemically reducible cytochrome b_h. This pool of cytochrome b_h is thus rapidly accessible to the PSII-formed PQH_2. Half of this pool is reduced by an approx. 3 ms light pulse. Much longer pulses (several hundreds of ms) are required to reduce the remaining fraction of cytochrome b_h. The half-time of cytochrome b_h reoxidation after long pulses (> 200 ms) is about 200 ms (data not shown). Thus, one expects

that the slow reduction phase of cytochrome b_h is truncated by cytochrome b_h reoxidation, which would explain why the amount of photo-reducible cytochrome b_h (about 0.60) is lower than the amount of chemically reducible cytochrome b_h (about 0.70).

Curve 2 shows the reduction of cytochrome b_h in the presence of 0.1 μM DCMU. At this DCMU concentration, about 86% of PSII centers were inhibited, as measured by fluorescence experiments in the presence of ferricyanide (not shown). Under these conditions, the amount of rapidly accessible cytochrome b_h is about 0.44 of that accessible during the same phase in the absence of DCMU.

In Fig. 5, redox changes of P700 and cytochrome f were measured as a function of the dark time following a 80 ms pulse of saturating light. This illumination time is sufficient to reduce the largest part of PQ localized in the grana regions, but not that of stroma regions [4]. Even in the presence of such a large excess of PQH_2, the reduction of P700 remains a relatively slow process, which displays two phases ($t_{1/2} \approx 20$ ms and $t_{1/2} \approx 1$ s, respectively). Reduction of cytochrome f occurs with a half-time of about 150 ms; it is worth pointing out that, despite the large excess of PQH_2 produced by the flash, only about 68% of cytochrome f is reduced in a 1 s dark period and probably less, if one takes into account that the cytochrome f signal is not corrected for the contribution of c-550. We cannot exclude that in the seconds' time range, a slow reduction of cytochrome f could compete with its reoxidation by ferricyanide.

In the experiment of Fig. 6, the flash-induced cytochrome b_h reduction was measured using chloroplasts in the presence of 6 mM sodium ascorbate.

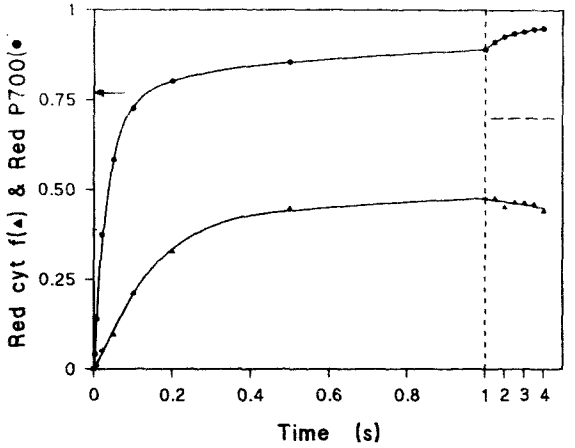

Fig. 5. Reduction of cytochrome f and P700 after an 80-ms saturating pulse. At time zero, cytochrome f and P700 are fully oxidized. Dashed line, total amount of photo-oxidizable cytochrome f (see Materials and Methods). Arrow, amplitude of the fast reduction phase of P700.

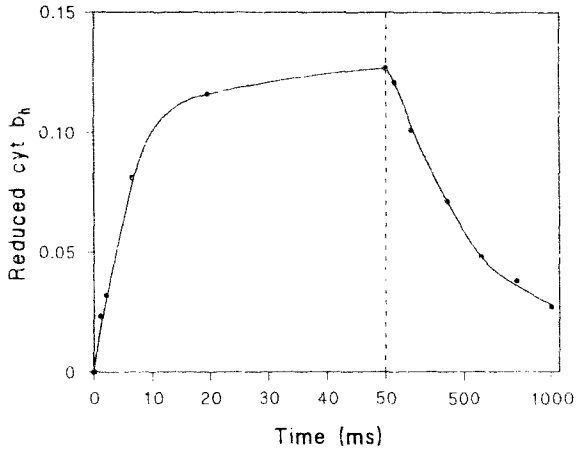

Fig. 6. Reduction of cytochrome b_h induced by non-saturating flashes given 2.7 s apart. 6 mM sodium ascorbate.

Chloroplasts were illuminated by a series of red (Schott filter RG695) non-saturating flashes spaced 2.7 s apart. This actinic light preferentially excites PSI, thus preventing accumulation of PQH_2 along the flash series. The half-time for cytochrome b_h reduction is about 4 ms, i.e., about 3-times shorter than the half-time measured in the presence of ferricyanide. The same experiment (not shown) was performed with saturating flashes and led to a somewhat slower cytochrome b_h reduction ($t_{1/2} \approx 7$ ms), which remains faster than under oxidizing conditions. We checked that, in the presence of a saturating concentration of DCMU, no cytochrome b_h was reduced upon the first or subsequent flashes. We also observed that a 2-s weak far-red pre-illumination, which oxidized a small amount of cytochrome f and a fortiori any PQH_2 present in the dark, did not modify the flash-induced reduction kinetics of cytochrome b_h. Thus, although PQH_2 involved in cytochrome b_h reduction in the presence of ascorbate arises from PSII turnover exclusively, the rate constant of cytochrome b_h reduction is about 3-times higher than under oxidizing conditions.

Discussion

Kinetics of reduction of cytochrome b_h and PSI donors

According to the the concerted reaction mechanism [13,14], oxidation of PQH_2 at site Q_Z leads to the transfer of one electron to the high potential chain (primary and secondary PSI donors) and one electron to the low potential chain (b cytochromes). In agreement with this hypothesis, reduction of cytochrome b_h and P700 during the initial phase occurs with same kinetics and amplitude (Figs. 2 and 3, curves 1 and 2). This implies a large equilibrium constant and fast electron transfer (< 2 ms) between cytochrome f and

P700. It implies, also, that most of the thylakoids have not lost plastocyanin during the chloroplast's preparation. This fast phase is close to an exponential, with a half-time (about 12 ms) independent of the amount of PQH_2 formed by the flash and of the fraction of cytochrome b_h oxidized prior to the flash (Figs. 1, 2 and 3). This behavior is characteristic of a first-order process limited by electron transfer reactions occurring within the cytochrome b/f complex.

At longer times than 20 ms after the flash, the number of electrons tranferred to the high potential chain becomes larger than that stored in cytochrome b_h. This implies that a second PQH_2 molecule is oxidized at site Q_Z in complexes including cytochrochrome b_h already reduced by a first PQH_2 molecule. This second concerted reaction leads to the transfer of a second electron to the high potential chain. As no accumulation of cytochrome b_l is observed, the second electron transferred to the low potential chain must trigger the reoxidation of cytochrome b_h and the formation of PQH_2 at site Q_C, as proposed in Ref. 14. If $n_1(t)$ is the number of PQH_2 oxidized by complexes including an oxidized cytochrome b_h, $n_2(t)$ the number of PQH_2 oxidized by complexes including a reduced cytochrome b_h, $n_c(t)$ the number of electrons stored in cytochrome b_h (Figs. 2 and 3, curve 1) and $n_d(t)$, the number of electrons stored in P700 and secondary PSI donors (Figs. 2 and 3, curve 3), one has: $n_c = n_1 - n_2$; $n_d = n_1 + n_2$ and thus, $n_1 = (n_c + n_d)/2$. If we assume that the probability of triple turnover is negligible, n_1 (plotted as curve 4, Figs. 2 and 3) represents the number of complexes in which one cytochrome b_h, and at least one PQH_2 molecule have been oxidized. Curve 4 displays two phases with half-times of 12 and 100 ms, respectively. The slow phase shows that a fraction of PQH_2 (about 1/3) has no rapid access to cytochrome b/f complexes. The amplitude of the fast phase is the same for curves 1 and 4 (Figs. 2 and 3) after extrapolation to time zero of the corresponding slow phases. In the case of curve 4, Fig. 3, a decay is observed at times beyond 1 s; this indicates that when PQH_2 is present in limiting amount, cytochrome b_h is reoxidized by a slow process ($t_{1/2} \approx 2$ s) not involving a second turnover at the Q_Z site, i.e., no electron transfer to the high potential chain.

We performed preliminary experiments (not shown) in the presence of 100 μM MOA-stilbene, known to inhibit site Q_Z in the cytochrome b/c complex [15] and preferentially site Q_C in the cytochrome b/f complex [16]. We observed that a group of 2 saturating flashes induced the reduction of cytochrome b_h and P700 with similar time-courses and amplitudes, at variance with what was observed in the absence of inhibitor (see Fig. 2, curves 1 and 2). Thus, the inhibition by MOA-stilbene of site Q_C prevents the oxidation of a second PQH_2 at site Q_Z and the oxidation of cytochrome b_h at site Q_C, as predicted by the model proposed above.

After dark adaptation in the presence of sodium ascorbate, the secondary donors are fully reduced while the PQ pool remains fully oxidized. Cytochrome b_h reduction in the presence of ascorbate, is about 3-times faster than in oxidizing conditions (Fig. 6), although in both conditions, PQH_2 exclusively arises from the PSII reaction. We conclude that, at least in oxidizing conditions, cytochrome b_h reduction is not limited by the rate of diffusion of PQH_2 to the cytochrome b/f complex or by the rate of binding of PQH_2 at the Q_Z-site. Therefore, as already stated, the rate of cytochrome b_h reduction, is limited by electron transfer reactions occurring within the cytochrome b/f complex. The rate of this limiting process is probably controlled by the redox state of the carriers of the high potential chain (Rieske protein, cytochrome f). Surprisingly enough, the highest rate of electron transfer is observed when the high potential chain is in its reduced state prior to the flash. A possible interpretation is that oxidizing or reducing conditions induce different conformational states and thus, different functional properties of the cytochrome b/f complex. Alternatively, it could be that positive charges stored in cytochrome f and the Rieske protein undergo electrostatic interactions with the charged semiquinone transiently formed at site Q_Z. This should increase the midpoint potential of the couple PQ/PQ^- and thus should decrease the rate of electron transfer to the low potential chain.

Most of these results can be interpreted in terms of a model already proposed in Refs. 4 and 5, in which diffusion of plastoquinone is restricted to membrane domains of limited size. Nevertheless, additional structural hypotheses are required to take into account some following paradoxical features: (1) the formation of at least 0.45 PQH_2 molecules by the second of a group of two saturating flashes (Fig. 2) induces the reduction of only 0.25 cytochrome b_h molecules during the fast phase ($t_{1/2} \approx 12$ ms), i.e., 63% of the rapidly accessible pool of cytochrome b_h (see Fig. 4); (2) when a limiting amount of PQH_2 is produced (Fig. 3), the reduction of cytochrome b_h displays a slow phase, which implies that a fraction of PSII-formed PQH_2 is not able to react rapidly with cytochrome b/f complexes, even when the amount of PQH_2 is much lower than that of the rapidly accessible cytochrome b_h.

Two classes of model can account for the data presented here: (1) the cytochrome b/f complex is associated with PSII reaction centers, forming supercomplexes. This hypothesis was already proposed by Lam and Malkin [17], who observed efficient electron transfer reactions between PSII and the cytochrome b/f complex in reconstituted systems in the absence of

added PQH_2. The formation of super complexes implies addition of Mg^{2+}, which is supposed to prevent the electrostatic repulsion between PSII and the cytochrome *b/f* complex, by shielding the negative charges present at the surface of this complex. From our experiments, we computed that, in the grana regions, there is about 0.45 cytochrome *b/f* complex per PSII center (as discussed below); thus, the supercomplexes should include 2 PSII centers and one cytochrome *b/f* complex, similar to those observed in *Rb. sphaeroides* R26 [18]. We assume that PQH_2 is released in a pocket including both the Q_B and Q_Z sites. Following the flashes, site Q_Z experiences a high transient local concentration of PQH_2. A major fraction of PQH_2 is rapidly trapped at site Q_Z and accounts for the first order kinetics of cytochrome b_h reduction. The remaining fraction of PQH_2 is released in the lipid phase of the membrane and reacts only slowly at site Q_Z. This model explains why, in Figs. 2 and 3, curves 4 display fast ($t_{1/2} \approx 12$ ms) and slow ($t_{1/2} \approx 100$ ms) phases, whatever the amount of PQH_2 produced, in excess (Fig. 2) or limiting (Fig. 3). According to curves 4, only about 66% PQH_2 produced is rapidly trapped at the Q_Z site. It has been previously estimated [4] that grana domains, in which PQH_2 diffusion is restricted, include an average number of four PSII centers, i.e., two supercomplexes; (2) no specific interaction between PSII and cytochrome *b/f* complexes is assumed. Plastoquinone diffuses rapidly within the isolated domains defined in Ref. 4. To account for the first-order kinetics of cytochrome b_h reduction, we must assume: (a) a fast equilibration (< 1 ms) of PQH_2 within the isolated domains; (b) a rapid binding (< 1 ms) to site Q_Z, and; (c) a great affinity of PQH_2 for site Q_Z. In this model, we expect a random distribution of PSII centers and cytochrome *b/f* complexes in the domains in which the diffusion of plastoquinone is restricted. This would lead to a highly variable stoichiometry [cytochrome *b/f*]/[PSII] within the different domains, with an average value of ~0.45. For the fraction of the domains in which $r > 0.8$, more than two flashes are required to produce enough PQH_2 to fully reduce cytochrome b_h. On the other hand, as the average number of cytochrome *b/f* per domain is small (about 2), the probability of having domains including PSII but no cytochrome *b/f* complexes is not negligible. PQH_2 formed in these domains would have access to cytochrome *b/f* complexes only via a leak between neighboring domains, which would occur in the 100 ms time-range. This leak would account for the slow phase in the reduction of P700, secondary PSI donors and cytochrome b_h, observed when a small amount of PQH_2 is produced (Fig. 3).

Cytochrome b/f complexes accessibility to PSII-formed plastoquinol

Structural and biochemical analyses [19–21] have led to estimates that the proportion of cytochrome *b/f* complexes located in the grana and stroma regions are about 2/3 and about 1/3, respectively. In the experiment of Fig. 4, the amount of cytochrome b_h reduced by pulses of saturating light is measured after completion of the electron transfer reactions occurring within the cytochrome *b/f* complexes. About 60% of cytochrome b_h is reduced by pulses of ~20 ms duration. It is very likely that this fraction of the cytochrome *b/f* complexes, rapidly accessible to PQH_2, is located in membrane domains including both PSII and cytochrome *b/f* complexes, i.e., in the grana regions. A 20-ms light pulse of saturating light generates enough PQH_2 to saturate all the Q_Z sites located in the grana regions. Much longer pulses are required to reduce the remaining fraction of cytochrome b_h, belonging to the cytochrome *b/f* complexes included in domains with no or few PSII centers, very likely in the stroma regions. This slow reduction of cytochrome b_h could arise from both the turnover of the few PSII centers present in these regions and of the diffusion of PQH_2 from grana to stroma regions.

In the presence of 0.1 μM DCMU, the rapidly accessible cytochrome *b/f* complexes represent only 47% of the control. If f is the fraction of inhibited PSII centers, n the number of centers per domain, the fraction of domains where all the centers are inhibited is $g = f^n$. In the experiment of Fig. 4,

$f = 0.86$, $g = 1 - 0.47 = 0.53$ and $n = 4$.

In these conditions, the relative amplitude of the slow phase of cytochrome b_h reduction is increased. This suggests that a leak between fully and partially inhibited domains occurs in the hundreds ms time-range.

Similar conclusions have been drawn previously from the measurement of the time-course of PQ reduction under saturating illumination [4]. (1) Reduction of the PQ pool displays two phases; a fraction of the pool (about 2/3) is reduced in less than 100 ms (spinach chloroplasts) and is very likely located in the grana regions. The remaining fraction is reduced in more than 2 s and corresponds to PQ located in the stroma region; (2) on the basis of experiments performed with partially inhibited centers, it has been concluded that rapid diffusion of PQ occurs in domains including an average of four PSII centers.

We have shown that an 80-ms light pulse, which produces a large excess of PQH_2 as compared to the amount of oxidized P700 and secondary PSI donors induces the reduction of only 68% of cytochrome *f* during the subsequent dark period (Fig. 5). Conversely, when P700 and secondary PSI donors are reduced (presence of sodium ascorbate, see Materials and Methods), a continuous illumination induces a full oxidation of cytochrome *f*. One can conclude that all of cytochrome *f* is accessible to positive charges arising

from PSI reaction, but that only 2/3 of cytochrome *f* (very likely located in the grana regions) is accessible to electrons arising from PSII reaction. This implies that plastocyanin is not able to mediate a rapid equilibration between cytochrome *b/f* complexes located in the grana and stroma regions, respectively. If such were the case, multiple turnovers of the cytochrome *b/f* complexes included in the grana regions would induce the reduction of cytochrome *f* present in the stroma regions. We do not yet have an interpretation which could account for this lack of equilibration.

Electron transfer reactions occurring at site Q_C

The mechanism of electron transfer reactions occurring at site Q_C is a subject of controversy as no reliable information is available on the potential of the PQ/PQ^- and PQ^-/PQH_2 couples at this site. On the basis of experiments performed with PSI-depleted mutants of *Chlorella sorokiniana* [22], we have been led to a tentative estimate of the midpoint potentials of these two couples. Illumination of the mutant by a saturating continuous light induced the reduction of 95% to 99% of the PQ pool and ~20% of cytochrome b_h by electron transfer reactions occurring exclusively at site Q_C. We then assume that the partial reduction of cytochrome b_h at site Q_C involves a one-electron process

$$PQH_2 + \text{cytochrome } b_h \rightleftharpoons PQ^- + \text{cytochrome } b_h^- + 2H^+$$

In agreement with this hypothesis, McGill et al. [23] observed in the presence of reduced quinone the formation of a weak free radical they ascribed to a semiquinone at site Q_C. To take into account that about 20% of cytochrome b_h is in its reduced state [22], the midpoint potential of the PQ^-/PQH_2 couple must be close to −10 mV. Consequently, the midpoint of the couple PQ^-/PQH_2 is about 230 mV, which implies that the oxidation of reduced cytochrome b_h by PQ is thermodynamically highly favorable. Then, under the oxidizing conditions used in this present work, the slow oxidation of cytochrome b_h we observed ($t_{1/2} \approx 2.5$ s) when the concentration of PQH_2 is much lower than that of the cytochrome *b/f* complexes, suggests that the affinity of PQ for site Q_C is very low. If such is the case, the cytochrome *b/f* complex will operate according to a semiquinone cycle [24], as already proposed in Ref. 22. It is worth pointing out that the values of the potentials of the PQ/PQ^- and PQ^-/PQH_2 couples we assumed imply a very high affinity of PQ^- for site Q_C. As the affinity of PQ^- for site Q_Z is low, the transfer of a semiquinone from Q_Z to Q_C sites should be a highly favorable process.

An alternative hypothesis, not considered in Ref. 22, is that the reduction of cytochrome b_h by PQH_2 at site Q_C involves a two-electron process. In the case of mitochondrial cytochrome *b/c* complexes, Marres and De Vries [25] assumed that these complexes are associated in functional dimers. Quinol induces at site Q_C the concerted reduction of the two cytochrome b_h of the dimer, through a two-electron process. In our experiments, reported in Ref. 22, the potential of the couple PQ/PQH_2 was 50 to 70 mV. If cytochrome *b/f* complexes were organized in functional dimers, one would expect a reduction of no more than 2% of cytochrome b_h, i.e., about 10 times lower than what we measured. Another possibility is that the PSII center and a monomeric cytochrome *b/f* complex form a supercomplex in which the PSII secondary acceptor Q_B and Q_C site share a common pocket. We then assume that PQH_2 is able to reduce both cytochrome b_h and Q_B, according to a two-electron process. As the midpoint potential of the couple Q_B/Q_B^- is about 80 mV, the concerted reduction of cytochrome b_h and Q_B will lead to the reduction of 10 to 20% of cytochrome b_h, a value compatible with our experimental results. This interpretation does not impose any constraint on the value of the potential of the PQ/PQ^- and PQ^-/PQH_2 couples at site Q_C. Then, the slow oxidation of cytochrome b_h observed in oxidizing conditions could be explained if the potential of the PQ/PQ^- couple at site Q_C is lower than that of cytochrome b_h. This interpretation is compatible with a Q-cycle mechanism. In this case, the reduction of PQ at site Q_C could occur through a two-electron process only when both cytochrome b_h and cytochrome b_l are in their reduced state.

Acknowledgements

The authors wish to thank J. Lavergne and W. Nitschke for their critical reading of the manuscript. This work was supported by Centre National de la Recherche Scientifique (URA 1187) and by Collège de France.

References

1 Whitmarsh, J. (1986) in Photosynthesis III, Encyclopedia of Plant Physiology (Staehlin, L.A. and Arntzen, C.J. eds.), Vol. 19 pp. 508–525, Springer Verlag.
2 Joliot, P., Lavergne, J. and Béal, D. (1990) in Current research in Photosynthesis (Baltcheffsky, M., ed.), Vol. II, pp. 879–882, Kluwer, Dordrecht.
3 Lavergne, J. and Joliot, P. (1991) Trends Bio. Sci. 16, 129–134.
4 Joliot, P., Lavergne, J. and Béal, D. (1992) Biochim. Biophys. Acta 1101, 1–12.
5 Lavergne, J., Bouchaud, J.-P. and Joliot, P. (1992) Biochim. Biophys. Acta 1101, 13–22.
6 Joliot, P. and Joliot, A. (1984) Biochim. Biophys. Acta 765, 210–218.
7 Joliot, P. and Joliot, A. (1984) Biochim. Biophys. Acta 765, 219–226.

8 Joliot, P., Béal, D. and Frilley, B. (1980) J. Chim. Phys. 77, 209–216.
9 Rich, P.R., Heathcote, P. and Moss, D.A. (1987) Biochim. Biophys. Acta 892, 138–151.
10 Ke, B. (1972) Arch. of Biochem. 152, 70–77.
11 Rolfe, S.A., Sanguansermsri, M. and Bendall, D.S. (1987) Biochim. Biophys. Acta 894, 434–442.
12 Kramer, D.M. (1990) PhD. Thesis
13 Mitchell, P. (1975) FEBS Lett. 59, 137–139.
14 Velthuys, B.R. (1979) Proc. Natl. Acad. Sci. USA 76, 2765–2769.
15 Von Jagow, G. and Link, T.A. (1986) Methods Enzymol. 126, 253–271.
16 Rich, P.R. (1991) Biochem. Soc. Trans. 19, 263.
17 Lam, E. and Malkin, R. (1983) Arch. Biochem. Biophys. 224, 2, 456–463.
18 Joliot, P., Verméglio, A. and Joliot, A. (1989) Biochim. Biophys. Acta 975, 336–345.
19 Staehlin, L.A. (1986) in Photosynthesis III, Encyclopedia of Plant Physiology (Staehlin, L.A. and Arntzen, C.J., eds.), Vol. 19 pp. 1–84, Springer Verlag.
20 Olive, J., Vallon, O., Wollman, F.-A., Recouvreur, M. and Bennoun, P. (1986) Biochim. Biophys. Acta 851, 329–348.
21 Vallon, O. Bulté, L., Dainese, P., Olive, J., Bassi, R. and Wollman, F.-A. (1991) Proc. Natl. Acad. Sci. USA 88, 8262–8266.
22 Joliot, P. and Joliot, A. (1988) Biochim. Biophys. Acta 933, 319–333.
23 McGill, J.W., Kumar, S. and Salerno, J.C. (1986) Biophys. J. 49, 484a, (WPos123).
24 Wikström, M. and Saraste, M. (1984) in Bioenergetics (Ernster, L., ed.), pp. 49–94, Elsevier Science Publishers, Amsterdam.
25 Marres, C.A.M. and De Vries, S. (1991) Biochim. Biophys. Acta 1057, 51–63.