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CYTOCHROME *f* AND PLASTOCYANIN KINETICS IN *CHLORELLA PYRENOIDOSA*

II. REDUCTION KINETICS AND ELECTRIC FIELD INCREASE IN THE 10 ms RANGE

BERNADETTE BOUGES-BOCQUET

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

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SUMMARY

On dark-adapted *Chlorella*, after one flash, plastocyanin (PC) undergoes reduction with a half-time of 7 ms. After 4 or 5 flashes, the reduction of PC^+ in the 10 ms range is suppressed, and the level of oxidized plastocyanin increases during the next few flashes before reaching a stationary value. Cytochrome *f* exhibits approximately the same pattern.

The reduction of PC^+ and cytochrome f^+ in the 10 ms range is correlated with an increase of the electrice field named phase b (Joliot, P. and Delosme, R., Biochim. Biophys. Acta 357 (1974) 267–284). Both need the presence of a compound R′ in the reduced state. A dark electron transfer involving a carrier of electrons across the membrane, a proton carrier, R′ as terminal reducant, PC^+ and cytochrome f^+ as terminal oxidants, would account for this field generation.

Cooperation between the electron transfer chains is implied at the level of plastocyanin oxidation. An equilibrium constant of about 2 is observed between cytochrome *f* and plastocyanin before 1 ms and after 500 ms after the photochemical reactions. We observe that cytochrome *f* and plastocyanin are not connected from 1 to 100 ms after a photochemical reaction. The equilibrium constant between plastocyanin and *P*-700 remains large [20] under these conditions.

INTRODUCTION

In the previous paper [1], we have presented a linear model which accounts for the oxidation kinetics of cytochrome *f* and PC on dark adapted *Chlorella*. However, this model cannot explain the many changes of apparent redox equilibrium constants which have been reported in the literature at the level of the system I

Abbreviations: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; PC, plastocyanin; DBMIB, 2,5-dibromo-3-methyl-6,isoproyl-1,4-benzoquinone; DAD, 2, 3, 5, 6 tetramethyl-*p*-phenylenediamine.

donors: changes between Q (primary acceptor of system II) and *P*-700 (primary donor of System I) [2], changes between plastoquinone pool and *P*-700 [3], changes between cytochrome *f* and *P*-700 [4]. Each of these systems exhibits a high equilibrium constant when observed in the dark, and a low equilibrium constant in the light. Another unexpected behaviour of these donors is presented by Haehnel [5]: cytochrome *f* reduction after far red light does not exhibit the lag which would be expected if the electrons were transferred with a high equilibrium constant from cytochrome *f* to *P*-700. In order to better understand these problems, we have studied the kinetics of PC and cytochrome *f* on whole algae illuminated with a series of flashes.

An absorption increase at 515 nm in the 10 ms range after a flash (phase b) has been observed [6, 7]. This phase corresponds to an increase of the electric field. It is linked to Photosystem I and disappears after a few flashes [7]. We will show that this field increase is correlated with the reductions of plastocyanin and cytochrome *f*.

MATERIALS AND METHODS

Chlorella Pyrenoidosa was grown on Knopp medium containing Arnon's trace elements A_5 et B_6. The preparation was illuminated by white fluorescent light of 3000 lux. The chlorophyll concentration was 30 μg/ml. For all the experiments, cells were dark adapted for 10 min.

Absorption changes were measured using the flash detector differential spectrophotometer described by Joliot and Delosme [7]. The actinic flashes were red having been filtered through two filters (Wratten 34 + Wratten 24). They excited 80 % of the centers of Photosystem I. Complementary filters (Schott BG 38) were placed in front of the photoelectric cells.

Plastocyanin was detected at 584 nm after subtraction of a small electrochromic effect. When System II was blocked, cytochrome *f* was detected by the difference of the absorptions at 545 and 553 nm after substraction of the electrochromic effect [1]. The electrochromic effect was detected at 515 nm [8].

RESULTS

In the previous paper [1], we have presented the kinetics of plastocyanin after one flash: the rise of the oxidation level to 200 μs is followed by a reduction with a half-time of 7 ms. If instead of one flash, a series of flashes is fired on dark adapted *Chlorella* cells, we observe no reduction of PC^+ in the 10 ms range after 5 or more flashes (Fig. 1). These measurements were made with Photosystem II blocked by hydroxylamine and DCMU [9], so that comparable results could be presented for cytochrome *f*. However, plastocyanin exhibits the same pattern in the absence of hydroxylamine and DCMU. The addition of 0.1 M NH_4Cl induces no changes in the PC kinetics, thus indicating that the absence of PC^+ reduction is not caused by a pH change.

The redox pattern of cytochrome *f* under the same conditions is rather similar (Fig. 2) (the insert only shows that we are indeed measuring the pattern of cytochrome *f*). Cytochrome *f* reduction in the 100 ms range however takes place after 6 or more flashes when plastocyanin reduction is much slower. The kinetics of cyto-

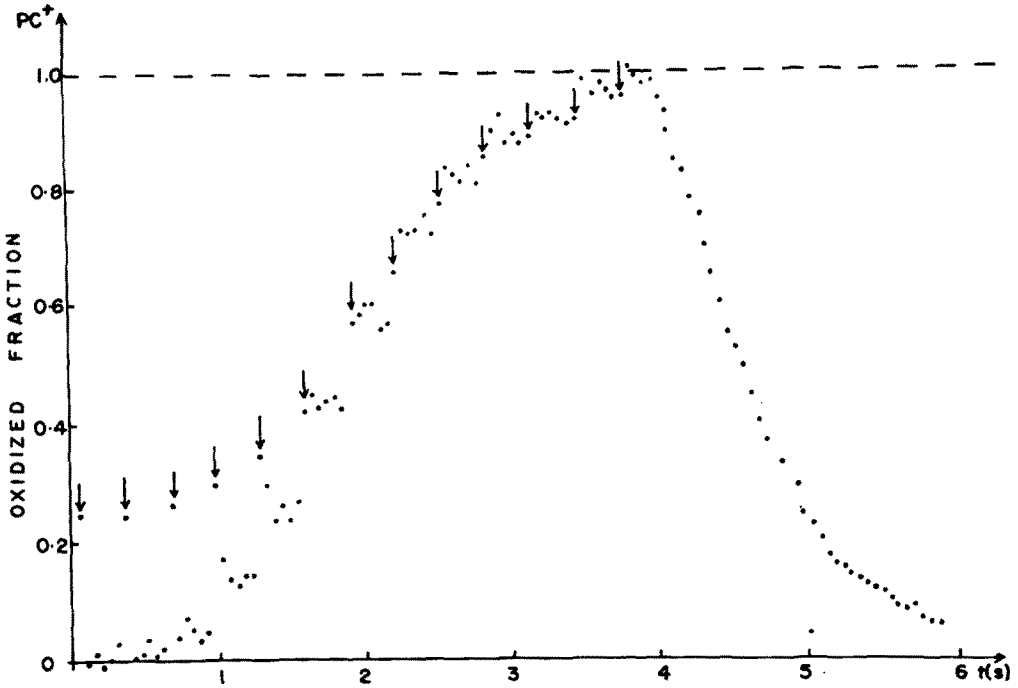


Fig. 1. PC changes upon illumination with a series of flashes. The time between two detecting flashes is 54 ms; one actinic flash is fired every 6 detecting flashes; the arrow indicates the detecting flashes fired 630 μs after an actinic flash. NH_2OH, 10^{-4} M; DCMU, 10^{-5} M.

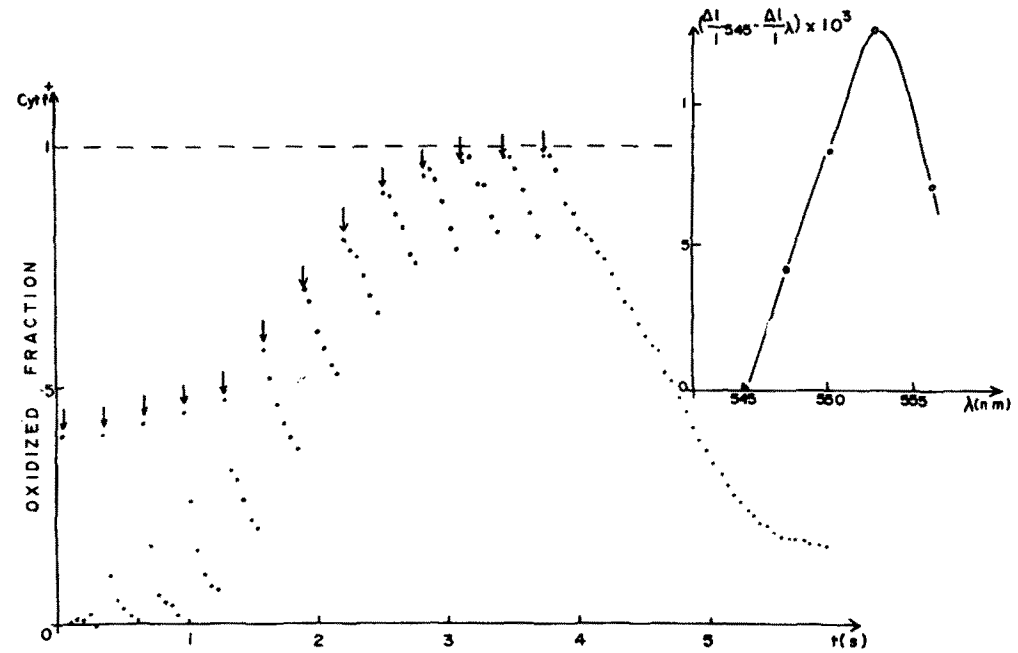


Fig. 2. Cytochrome *f* changes upon illumination with a series of flashes. Same conditions as for Figure 1. Insert: Difference of absorption changes $(\Delta I_{545}/I_{545})-(\Delta I_\lambda/I_\lambda)$ (after subtraction of the electrochromic effect) at 3 s after the beginning of a series of flashes. Same conditions as for Fig. 1.

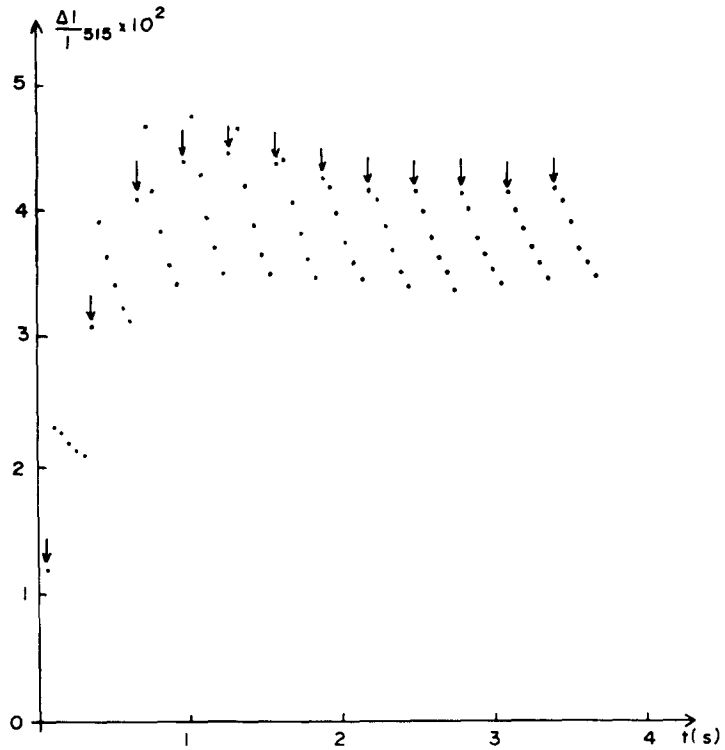


Fig. 3. Absorption changes at 515 nm upon illumination with a series of flashes. Same conditions as for Fig. 1.

chrome *f* cannot be obtained in the absence of hydroxylamine and DCMU because of overlapping changes due to C-550 [10].

The highest levels of oxidized plastocyanin and cytochrome *f* which can be reached photochemically were estimated upon illumination with strong light (one flash every 50 ms) with System II blocked. The amounts of plastocyanin and cytochrome *f* oxidized after 1 flash on dark adapted *Chlorella* were respectively 25 and 40 % of the total amounts which can be photochemically oxidized.

We saw that the fast reductions of both plastocyanin and cytochrome *f* in the 10 ms range disappear after a few flashes (Figs. 1 and 2). The slow electric field increase (phase b) also disappears after a few flashes (Fig. 3). The similarity between the rates of PC^+ reduction after the first flashes and of the phase b rise [7] leads us to look for additional correlations.

The next experiment was done in the absence of hydroxylamine and DCMU, and thus, the electrons were transferred from System II to System I. The field decay is slowed down by the addition of 10^{-4} M tri-*N*-butyltin-chloride (Diner, B., unpublished results) to allow a better observation of phase b. Phase b and PC^+ reduction disappear in the presence of an oxidant (Table I) but both are restored with the addition of a reductant. 10^{-5} M dibromothymoquinone (DBMIB) also inhibits phase b and PC^+ reduction, but in this case, no restoration is observed after the

TABLE I

PHASE B AND PC^+ REDUCTION IN THE 10 ms RANGE ON DARK ADAPTED *CHLORELLA* CELLS WITH 10^{-5} M TBT

	Additions	Phase b	PC^+ PC
10th flash	None	−	−
1st flash	None	+	+
	DAD $5 \cdot 10^{-4}$ M $K_3Fe(CN)_6$ $2 \cdot 10^{-5}$ M	−	−
	DAD $5 \cdot 10^{-5}$ M $K_3Fe(CN)_6$ $2 \cdot 10^{-4}$ M $Na_2S_2O_4$ $2 \cdot 10^{-2}$ M	+	+
	DBMIB 10^{-5} M	−	−
	DBMIB 10^{-5} M $Na_2S_2O_4$ $2 \cdot 10^{-2}$ M	−	−

addition of a reductant. Trebst and Harth [11] have shown that DBMIB, a quinone antagonist, inhibits the electron transfer from the plastoquinone pool to the System I donors, on chloroplasts. This inhibition is also observed on *Chlorella* [12] but only during a few minutes after its addition (Bennoun, Diner and Trebst, personal communication). Via the study of the fluorescence gush [13], we verified that 10 min after the addition of 10^{-5} M DBMIB, the electrons are transferred again from System II to System I. Under these conditions PC^+ reduction and phase b after the first flash are inhibited, thus indicating that the inhibitor effect of DBMIB on PC^+ reduction and phase b is different from the inhibitory effect of DBMIB on the plastoquinone pool.

In order to determine the relationship between PC and cytochrome *f*, we have studied more precisely the kinetics of these two components after the first, fifth and ninth flash of a series from 0 to 50 ms after the flash (Fig. 4). The base line for these kinetics is the level of oxidation just before the flash. The oxidation rate of plastocyanin is the same after the first and the fifth flashes, but slows down after the ninth flash. An oxidation of plastocyanin and a reduction of cytochrome *f* can be observed at the same time after the fifth and the ninth flash. After the ninth flash, no lag is observed in cytochrome *f* oxidation. If the frequency of the flashes of the series is more rapid, the same kinetics are observed but the amounts of cytochrome *f* oxidized after the fifth and the ninth flashes are smaller (not presented on a figure).

DISCUSSION

Reactions in the 10 ms range

The correlations between PC^+ reduction before 50 ms and phase b (Figs. 1 and 3, Table I) make it reasonable to assume that the two phenomena are linked. Both are suppressed in the presence of an oxidant and restored by the addition of a reductant (Table I), indicating that both need the presence of a compound R′, in the reduced state. R′ could be the reductant R put forward by Diner and Mauzerall [14] in *Chlorella*, which does not exist in chloroplasts. The field would be generated

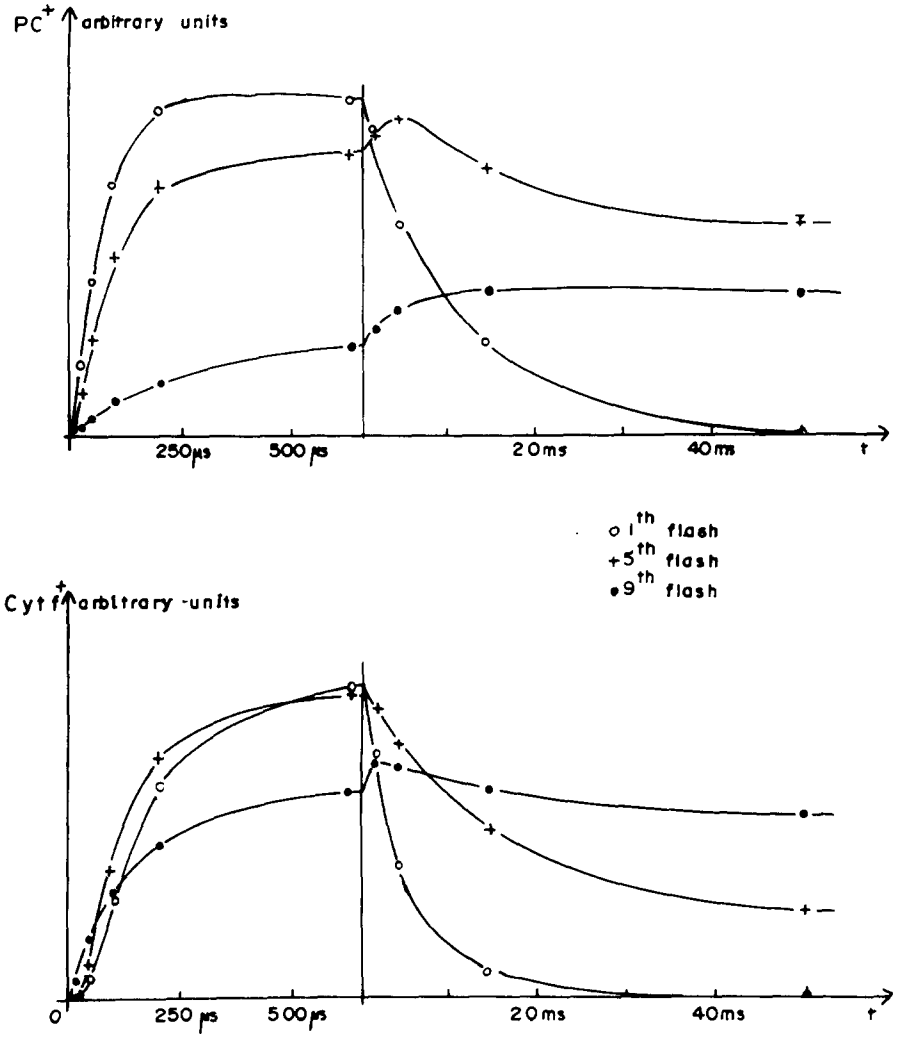
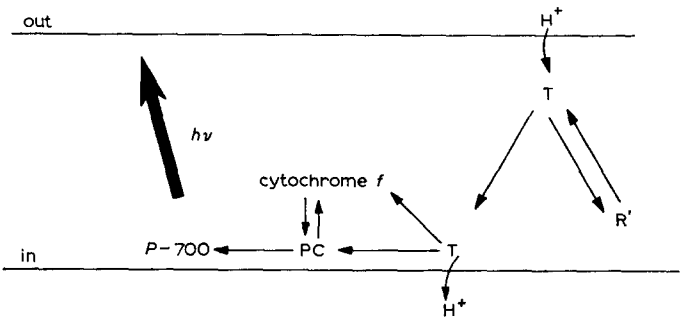


Fig. 4. PC and cytochrome *f* changes after the first, the fifth and the ninth flash of a series of frequency 3 Hz. The zero level is the level of oxidation just before the flash. NH_2OH, 10^{-4} M; DCMU, 10^{-5} M.

by the transfer from reductant R′ to plastocyanin and cytochrome *f*. This interpretation differs from the one proposed on bacteria [15] where a double loop, across the membrane, of the electron transfer chain including the cytochromes was proposed. Since R′ is neither in the transfer chain between System II and System I, nor in the cyclic transfer chain (disappearance of phase b after a few flashes), a model of double loop across the membrane of the main electron transfer chain [16] does not seem probable.

The field generation is slightly slower than PC^+ and cytochrome f^+ reduction, indicating that PC^+ and cytochrome f^+ reductions are not the electrogenic reactions. The simplest model to interprete a field generation in absence of a charge separation

is a sequence of an electron carrier across the membrane followed by a proton carrier (T). So the following scheme could explain phase b:



An equilibrium between two electrons carriers across the membrane would allow PC^+ and cytochrome f^+ reductions to "draw" electrons across the membrane, thus generating a field in the 10 ms range.

The inhibition of this electron transfer chain by the quinone antagonist DBMIB suggests that the proton carrier T could be a quinone. The inactivation of this chain after a few flashes could be due to a slow rate of regeneration of the reductant R' (in this case estimated to a few seconds). If, as we will suggest below, there are no connections between PC and cytochrome *f* from 1 ms to 100 ms after a photochemical reaction, both PC^+ and cytochrome f^+ would act as terminal oxidant of the chain.

P-700, PC and cytochrome f relations

In a previous paper [1], we showed that the lag of cytochrome *f* oxidation after one flash strongly argues in favor of a linear scheme for the electron transfers from *P*-700 to PC and cytochrome *f*. Even though, a parallel scheme where both PC and cytochrome *f* gives electrons to *P*-700 could explain this lag if additionally a switch between *P*-700 and cytochrome *f* is assumed: cytochrome *f* would become connected to *P*-700 in 50 μs after a flash. The absence of delay in cytochrome *f* oxidation after the ninth flash (Fig. 4), under conditions where some oxidized plastocyanin is accumulated (Fig. 1), is consistent with the linear scheme presented in ref. 1 but would require new additional assumptions to be interpreted in a parallel scheme. So, the parallel scheme is very improbable and the following discussion will take place in the frame of the linear model.

The slower rate of oxidation of PC when some oxidized plastocyanin is accumulated indicates a cooperation between donors at the level of the reaction P^+-700+PC → PC^++*P*-700: the higher the level of PC^+, the slower the rate of this reaction. Such a cooperation has already been put forwards on chloroplasts [17].

Taking into account that the field increase after a flash is a test of the quantity of reduced *P*-700 before the flash, and comparing Figs. 1 and 3, we compute an equilibrium constant of about 20 between *P*-700 and PC. This is consistent with the results of Marsho and Kok [14] and Haehnel [18].

An equilibrium constant of 2 was computed between cytochrome *f* and plastocyanin during the first millisecond after one flash [1]. The same equilibrium constant

is computed 600 μs after each flash of a series of flashes spaced from 300 ms (Figs. 1 and 2). But below 1 ms, the reduction of cytochrome *f* which is observed during the oxidation of plastocyanin (Fig. 4) is incompatible with this equilibrium constant.

Using an apparent equilibrium constant which is defined as (cytochrome f^+) (PC)/ (cytochrome *f*) (PC^+) we nonetheless observe that in a few hundred milliseconds after a flash, this quantity comes back to 2 and remains close to 2 at longer dark times (from Figs. 1 and 2).

The anomaly between one and a few hundred milliseconds can be explained by two hypotheses: (1) The redox potential of cytochrome *f* is changed. Such changes in the potential have already been put forward for cytochrome b_6 [19] and for cytochrome b_{559} [20]. But, if only the cytochrome *f* potential changed, the equilibrium constant between the plastoquinone pool and *P*-700 would not be affected. Since a change in the apparent equilibrium constant between the plastoquinone pool and *P*-700 is observed upon illumination [3], we favor rather the next hypotheses. (2) The connection between cytochrome *f* and plastocyanin is interrupted after a photochemical reaction. An equilibrium constant of 2 between cytochrome *f* and PC more than 500 ms after a photochemical reaction, no connections between cytochrome *f* and PC from 1 to 100 ms after a photochemical reaction, and an equilibrium constant of 20 between PC and *P*-700, explains the results of Malkin [21], of Marsho and Kok [14] and Haehnel [5, 18] in one single description. Moreover, Fig. 4 shows that the transition from an equilibrium constant of 2 to the absence of an equilibrium constant takes place 1 ms after the photochemical reaction. The time necessary for this transition could be consistent with a conformational or structural change of either cytochrome *f* or the membrane. A change in the accessibility of cytochrome *f* to external oxidants induced by light has been put forward by Horton and Cramer [22] but, as the latter change is stable in the dark for a few minutes, we think that these are two independent phenomena. Within the frame work of this hypothesis, the change in the apparent equilibrium constant between PQ and *P*-700 [3] is explained if it is assumed in addition that the electron transfer from plastoquinone to plastocyanin takes place via cytochrome *f*. This last assumption is consistent with the results of Wood and Bendall [23]. Our results can be summarized on the following scheme:

PQ ⇆ cytochrome *f* ⇆ PC ⇆ *P*-700

↓ 1 ms after a photochemical reaction

PQ ⇆ cytochrome *f* PC ⇆ *P*-700

↓ a few hundred milliseconds in the darkness

PQ ⇆ cytochrome *f* ⇆ PC ⇆ *P*-700

When the flash frequency is more rapid, the amounts of cytochrome *f* oxidized by the flashes are smaller but the kinetics remain unchanged. This result is consistent with the above scheme: the flashes are then fired when cytochrome *f* is partly disconnected from plastocyanin.

Upon illumination, an analoguous change of the apparent equilibrium constant has been observed between Q and the plastoquinone pool [24]. An assumption of

disconnection between Q and plastoquinone has been proposed [25]. The biological meaning of such disconnections in the electron transfer chain is unclear.

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