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

I. OXIDATION KINETICS AFTER A FLASH

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SUMMARY

P-700, plastocyanin and cytochrome *f* redox kinetics were measured after one flash, using dark-adapted *Chlorella* in the presence of hydroxylamine and 3(3,4-dichlorophenyl)-1,1-dimethylurea. Plastocyanin becomes increasingly oxidized with a half-time of 70 μs, then undergoes reduction with a half-time of 7 ms. Cytochrome *f* oxidation has a sigmoidal time-course and a half-time of 100 μs. Its reduction exhibits a half-time of 4 ms. These results are interpreted in a linear scheme:

$$P^+\ 700 + PC \xrightarrow{4.6 \cdot 10^3\ s^{-1}} P\ 700 + PC^+$$

$$PC^+ + \text{cytochrome } f \underset{7 \cdot 10^3\ s^{-1}}{\overset{14 \cdot 10^3\ s^{-1}}{\rightleftharpoons}} PC + \text{cytochrome } f^+$$

An equilibrium constant of 2 between cytochrome *f* and plastocyanin (PC), which contrasts with the large equilibrium constant between PC and *P*-700 is computed.

The presence of cytochrome b_6 in a cyclic path around Photosystem I is confirmed under these conditions.

INTRODUCTION

Three different electron donors have been observed in Photosystem I:

(1) *P*-700 is the primary donor of Photosystem I [1]. The oxidation and reduction of *P*-700 have been detected by spectroscopy [1] and by electron spin resonance measurements [2].

(2) Cytochrome *f*, first investigated by Duysens et al. [3], is always monitored by spectroscopic measurements.

(3) The oxidation of plastocyanin (PC) by Photosystem I was demonstrated by de Kouchkovsky and Fork [4] and by Gorman and Levine [5]. It can be detected

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Abbreviations: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; PC, plastocyanin.

either by spectroscopy [4] or by electron spin resonance [6].

No other donors have been detected between photoreaction I and the limiting step which separates the plastoquinone pool from Photosystem I. The relationship between these donors is a controversial subject [7–11] and no satisfactory model has as yet been proposed. In this article, we describe coordinated measurements of *P*-700, PC and cytochrome *f* which have given us new information on this question.

MATERIALS AND METHODS

Cholorella pyrenoidosa was grown on Knopp medium to which were added Arnon's trace elements A_5 and B_6; the preparation was illuminated by white fluorescent light of 3000 lux.

For all the experiments, Photosystem II was blocked by a preillumination in the presence of hydroxylamine (10^{-4} M) and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (10^{-5} M) [12]. The cells were then dark-adapted for 10 min. The chlorophyll concentration was 30 μg/ml.

Absorption changes were measured, using the flash detector differential spectrophotometer described by Joliot and Delosme [13]. The actinic flashes were red, having been filtered through two filters (Wratten 34 and Wratten 24). They excited 80 % of the centers of Photosystem I (except in the case shown in Fig. 1 where only Wratten 24 was used and where the flashes were saturating). Complementary filters (Schott BG 38) were placed in front of the photoelectric cells. Because the high intensity of fluorescence makes it impossible to measure the *P*-700 absorption changes in the 700 nm region with this technique, we used another method: since the recovery of the photochemical activity of system I after a flash follows the reduction kinetics of P^+-700 as measured by Haehnel [14], then, if two saturating flashes are separated by time t, the field generated by the second flash is a measure of reduced *P*-700 at time t after the first flash [13].

Cytochrome *f* was detected in its chemical difference absorption band around 553 nm. This narrow band presents two isobestic points at 544 and 560 nm (for an example, see ref. 5). When system II was blocked, three other absorption changes have been observed in this region: the electrochromic effect [15], the plastocyanin change [16] and the *P*-700 change [17]. Since plastocyanin and *P*-700 present almost flat spectra between 544 and 560 nm, a differential method can be used to obtain cytochrome *f* kinetics after subtraction of the electrochromic effect. The kinetics of the electrochromic effect were detected at 515 nm.

Katoh et al. [16] have shown that the chemical difference spectrum of plastocyanin has a broad band around 595 nm. In this range, an absorption change due to *P*-700 [17] with an isobestic point at 584 nm is also observed [10]. Thus, at 584 nm, the absorption change is mainly due to plastocyanin.

All the experiments presented in this paper have been done several times, and the results were very reproducible.

RESULTS

The kinetics of the reduction of P^+-700 after one flash (Fig. 1, curve 1) are in agreement with the kinetics presented by Haehnel and Witt [14]. At 630 μs, 70 %

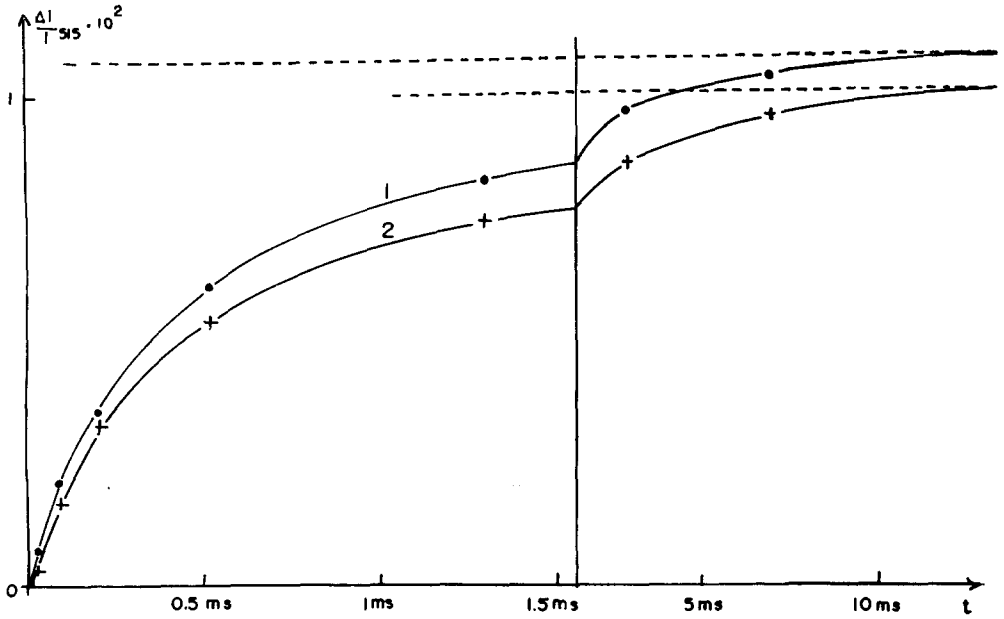

Fig. 1. Recovery of the photochemical activity of System I at time t, detected by the 515 nm absorption change observed 100 μs after a flash given at time t: (1) Recovery after one flash; (2) recovery after the second of the two flashes separated by 630 μs. The asymptotic lines were obtained when t = 320 ms.

of P^+-700 has been reduced and the reactions with the plastoquinone pool which reacts more slowly [18, 19], have not yet taken place. A recovery measured after the second of two flashes separated by 630 μs (Fig. 1, curve 2) is close to the first one, indicating that more than one rapid donor is present between P-700 and the plastoquinone pool.

On *Chlorella* cells, it is possible to kinetically label the electrochromic effect with several chemicals including NH_2OH and tri-*N*-buthyltin-chloride, which slow down the field decay (Diner and Joliot, [27] and Diner, unpublished data). We have observed that, on dark adapted *Chlorella* cells, for times greater than 50 ms after a flash, at every wavelength between 544 and 600 nm, the absorption changes present the same kinetics as the electrochromic effect measured at 515 nm, whatever the field decay-time is. This means that the other reactions (P^+-700, PC^+ and cytochrome f^+ reductions) are over at 50 ms. Knowing the electrochromic effect at 50 ms at every wavelength and the kinetics of this effect (drawn at 515 nm, Fig. 4a), and assuming that the correlation is true for times shorter than 50 ms, we can compute the absorption change due to electrochromic effect at every time and at every wavelength. Thus, the electrochromic effect can be subtracted from any measument.

Fig. 2a presents the spectrum of the absorption changes $\Delta I_{544}/I_{544} - \Delta I_\lambda/I_\lambda$ at different times after one flash; Fig. 2b presents the same phenomenum but after correction of the electrochromic effect. This narrow assymetrical band which peaks at 553 nm is characteristic of cytochrome f [5]. Thus, after correction of the electrochromic effect, $\Delta I_{544}/I_{544} - \Delta I_{553}/I_{553}$ is a pure measure of the cytochrome f redox state and its kinetics can be drawn (Fig. 2c). The half-time (approx 100 μs)

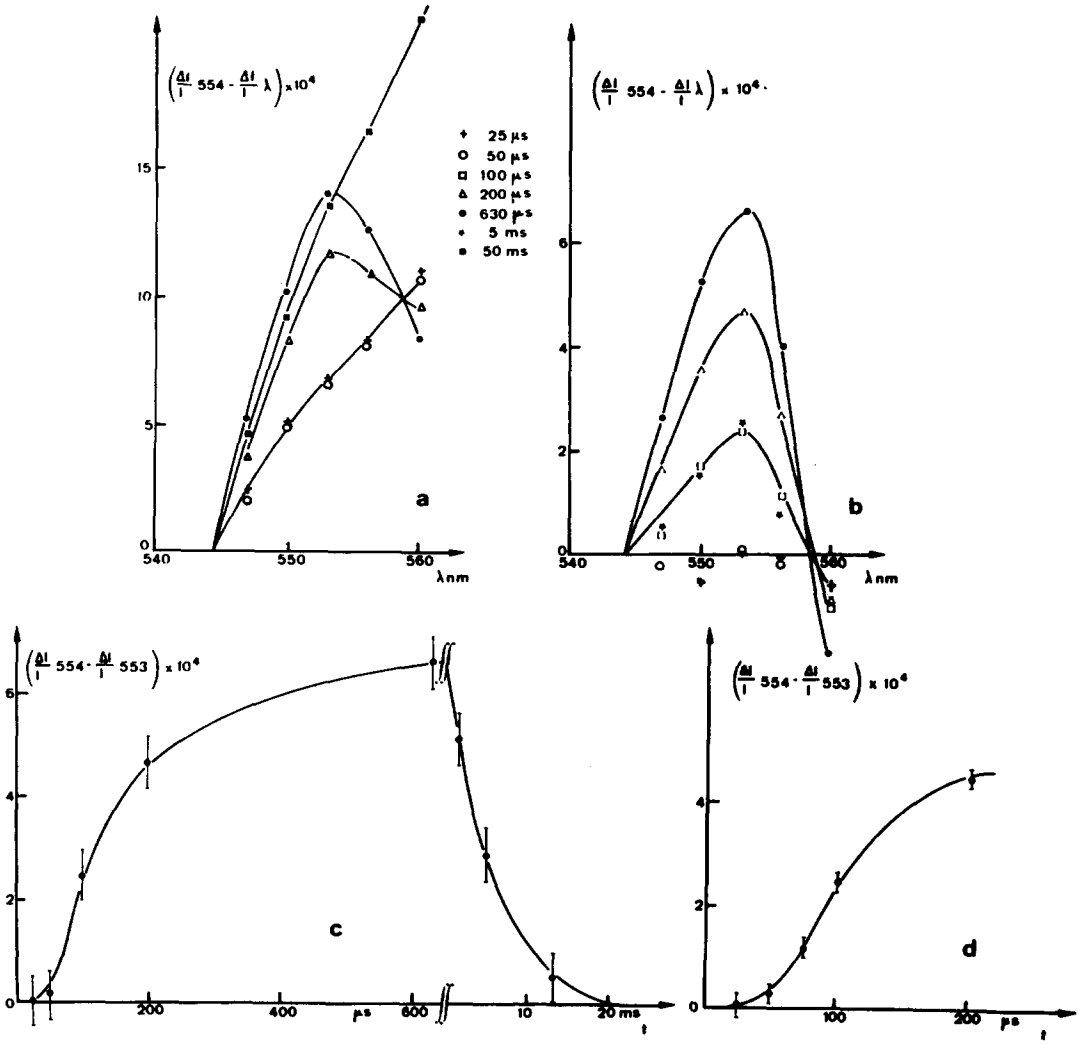

Fig. 2. (a) and (b) Difference of absorption change, $(\Delta I_{544}/I_{544})-(\Delta I_\lambda/I_\lambda)$ as a function of the wavelength at different times after one flash. (a) no corrections. (b) After substraction of the electrochromic effect. (c) Difference of absorption changes $\Delta I_{544}/I_{544}-\Delta I_{553}/I_{553}$ after subtraction of the electrochromic effect, as a function of the time after one flash. (d) Same as (c) but four times more integration, allowing the reduction of the error margin by a factor 2.

of cytochrome *f* oxidation is consistent with that measured by Hildreth [20]. The beginning of cytochrome *f* kinetics has been observed with smaller error margin (Fig. 2d): the oxidation kinetics clearly exhibit a sigmoidal shape.

Between 560 and 600 nm, three components (electrochromic effect, PC and *P*-700) contribute to the absorption change. The kinetics and spectra of these components have been obtained as follows:

(1) The electrochromic effect is measured at 50 ms, and thus, the spectrum of this effect can be drawn (Fig. 3a). Its kinetics (Fig. 4a) are observed at 515 nm. The

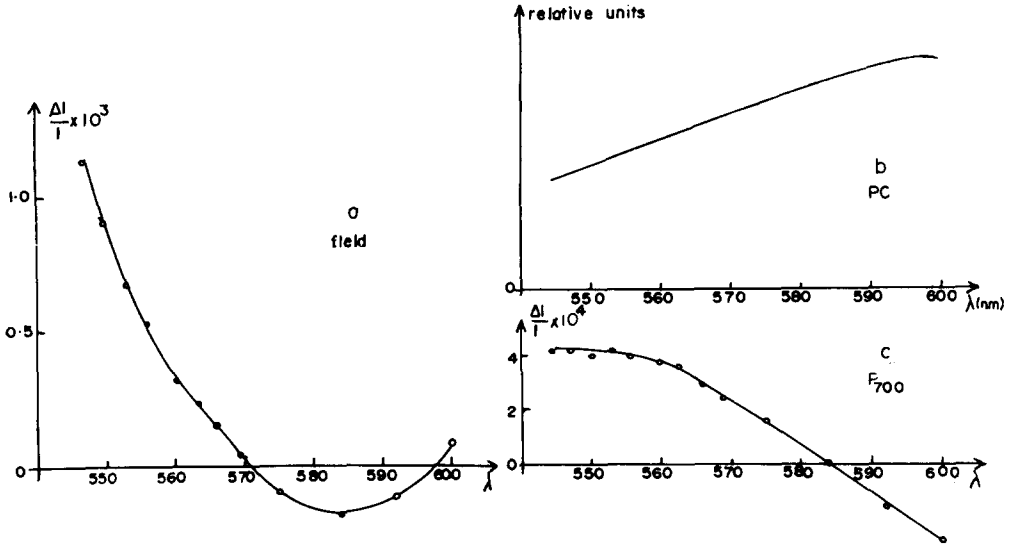

Fig. 3. (a) Absorption change at 50 ms after one flash, as a function of the wavelength. (b) chemical difference spectrum of parsley plastocyanin in phosphate buffer at pH 6.4. (c) absorption change at 25 μs after one flash, after substraction of changes due to field and to plastocyanin.

increase in absorption at 515 nm between 630 μs and 50 ms has already been observed by Witt and Moraw [21], Joliot and Delosme [13].

(2) We assume that after substraction of the electrochromic effect, the change at 584 nm is due only to the kinetics of plastocyanin (Fig. 4b, continuous line); the dotted line on Fig. 4b represents the change at 584 nm without any corrections. It can be noticed that at this wavelength, the correction due to electrochromic effect is small. PC becomes increasingly oxidized up to 200 μs after which the reduction of PC^+ is observed. This is consistent with the results of Haehnel [24]. The chemical difference spectrum of plastocyanin (Fig. 3b) from in vitro measurements of parsley plastocyanin in phosphate buffer at pH 6.4 has kindly been provided by R. P. Cox.

(3) The spectrum of the change remaining at 25 μs after subtraction of the electrochromic effect and of the small change due to plastocyanin is presented on Fig. 3c. It ressembles the spectrum observed by Ke [17] for *P*-700. The kinetics of *P*-700 are drawn from the recovery kinetics of the photochemical activity (Fig. 4c).

The amounts of plastocyanin and cytochrome *f* oxidized 630 μs after one flash on dark-adapted *Chlorella* cells are respectively 25 and 40 % of the total amounts which can be photochemically oxidized upon illumination with saturating light (ref. 25, Fig. 1 and 2). When the same flashes with the same concentrations of *Chlorella* cells in the cuvette are used, these amounts are reproducible. As the flashes excite 80 % of the centers of Photosystem I, and as 10 % of P^+-700 is reduced in the first 25 μs (fig. 4c), 70 % of oxidized P^+-700 remains 25 μs after a flash.

We have found the following absorptions: at 553 nm, $\Delta I/I = 4.15 \cdot 10^{-4}$, 25 μs after a flash (Fig. 3c) due to 70 % of oxidized *P*-700; from 544 to 553 nm, $\Delta I/I = 6.7 \cdot 10^{-4}$, 630 μs after a flash (Fig. 2c) due to 40 % of oxidized cytochrome *f*; at 584 nm, $\Delta I/I = 7.4 \cdot 10^{-4}$, 630 μs after a flash (Fig. 4b) due to 25 % of oxidized

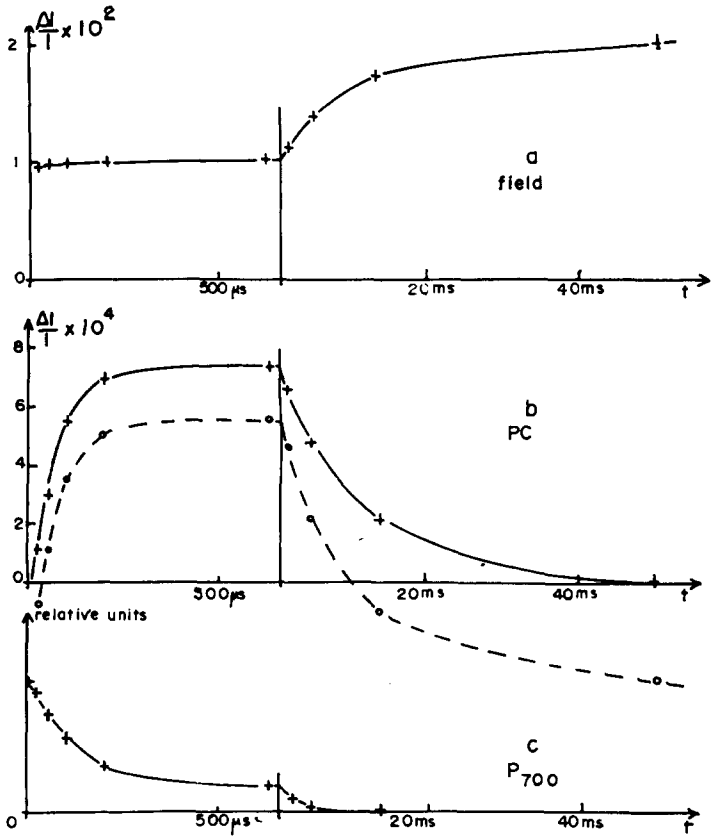

Fig. 4. (a) Absorption change at 515 nm as a function of the time after one flash. (b) absorption change at 584 nm as a function of the time after one flash, after substraction of the change due to the electrochromic effect (solid line) or with no corrections (dotted line). (c) recovery of the photochemical activity of System I at time t after one flash detected by 515 nm absorption change, observed 100 μs after one flash given at time t.

plastocyanin. Extinction coefficients of 6 $mM^{-1} \cdot cm^{-1}$ at 553 nm for P^+-700 [17], 16 $mM^{-1} \cdot cm^{-1}$ from 544 to 553 nm for cytochrome f^+ [26] and 5 $mM^{-1} \cdot cm^{-1}$ at 584 nm for PC^+ [16] have been observed. Our results thus indicate a stoichiometry of 1 : 6 : 1 for photooxidizible *P*-700, PC and cytochrome *f* in *Chlorella* cells.

Table I presents the absorption changes between 560 and 600 nm after one flash. It will be analyzed in the discussion.

DISCUSSION

To check whether the absorption changes between 560 and 600 nm can indeed be decomposed into three components (electrochromic effect, PC and *P*-700) we have computed the theoretical absorption changes between 560 and 600 nm as a sum of the kinetics presented on Fig. 4, taking into account the spectra presented on Fig. 3 (Table I, italic numbers). The difference between the experimental and the

TABLE I

ABSORPTION CHANGES, $(\Delta I/I) \cdot 10^4$, AS A FUNCTION OF THE WAVELENGTH AND OF THE TIME AFTER ONE FLASH

The straight numbers represent the experimental changes, the italic numbers, the computed changes (for more details, see text). On the last line, experimental uncertainties at each wavelength are reported.

Time after one flash	Wavelength							
	560 nm	563 nm	566 nm	569 nm	575 nm	584 nm	592 nm	600 nm
25 μs	7.3	7.4	4.3	3.6	1.5	−0.8	−2.2	−0.8
	7.8	*6.9*	*5.2*	*3.6*	*1.6*		*−1.7*	*−0.8*
50 μs	7.2	8.2	5.5	4.2	3.3	1.2	−0.7	2.7
	8.1	*7.4*	*6.0*	*4.6*	*2.9*		*−1.0*	*2.2*
100 μs	7.6	7.7	6.6	5.9	5.9	3.7	3.9	5.8
	8.1	*8.1*	*7.6*	*6.4*	*5.0*		*4.2*	*5.6*
200 μs	7.0	7.4	8.0	6.7	7.3	5.1	5.5	6.8
	7.6	*8.1*	*8.2*	*7.2*	*7.0*		*6.1*	*7.7*
630 μs	8.2	8.3	9.4	7.3	6.6	5.5	6.1	9.1
	6.8	*7.7*	*8.3*	*7.3*	*6.2*		*6.7*	*8.6*
1.70 ms	8.4	10.3	11.0	7.2	5.0	4.6	5.6	8.1
	6.5	*7.0*	*7.4*	*6.3*	*5.2*		*6.0*	*8.2*
5 ms	8.3	11.5	9.2	6.8	3.8	2.2	2.7	6.3
	7	*6.7*	*6.1*	*4.8*	*3.5*		*3.7*	*6.6*
13.5 ms	6.7	6.5	6.5	2.1	0.7	−1.1	−0.3	3.3
	6.6	*5.5*	*4.2*	*2.5*	*0.4*		*0.4*	*3.9*
50 ms	6.0	4.8	3.1	8.0	−1.2	−3.6	−2.3	1.7
	6.5	*4.8*	*2.9*	*8.0*	*−1.8*		*−2.3*	*1.7*
uncertainties	1.0	1.0	1.0	1.0	1.0	0.65	1.0	1.0

computed changes never exceeds the experimental errors, except at 1.70 and 5 ms, and from 560 to 569 nm. The consistent under-estimation of the absorption change in this region is very probably due to a reduction followed by reoxidation of cytochrome b_6 (see typical chemical difference spectrum of cytochrome b_6 in ref. 22, where the narrow band peaks at 563 nm). The maximum reduction would be around 5 ms. This would be consistent with the localisation of cytochrome b_6 in cyclic electron transport around Photosystem I [22]. The rate of cytochrome f^+ reduction and the rate of cytochrome b_6^- oxidation could be consistent with an oxidation of cytochrome b_6^- via cytochrome f^+ [23].

The above mentioned equality between experimental and computed changes indicates that the spectra and kinetics presented on Fig. 3 and 4 are correct: in particular, the change at 584 nm after substraction of the electrochromic effect, is purely due to plastocyanin.

There are three possible models for the connections of cytochrome *f*, plastocyanin and *P*-700:

(1) Cytochrome *f* and plastocyanin are not present in the same centers; (2) cytochrome *f* and plastocyanin are connected to *P*-700 in a linear chain; (3) cytochrome *f* and plastocyanin are connected to *P*-700 in parallel.

The sigmoidal shape of the cytochrome *f* oxidation time-course argues in favor of the linear scheme for electron transfer

cytochrome $f \rightarrow$ PC $\rightarrow$ *P*-700

To test the linear scheme further, and to compute the rate constants, we assumed that all the reactions are of the first order. For times greater than 1 ms after one flash, a large equilibrium constant can be computed between *P*-700 and PC (Fig. 5a) which is consistant with the constant $K = 20$ observed on chloroplasts [8, 10]. The kinetics of *P*-700 (half-time, 150 μs) then fits with a constant rate:

$k_1 = 4.6 \cdot 10^3\ s^{-1}$ for reaction P^+-700 PC $\rightarrow$ *P*-700 PC^+

The concentration of PC^+ reached 200 μs after one flash is 25 %. At each assumed equilibrium constant between PC and cytochrome *f* are associated a couple of rate constants k_2 and k_3 for the reactions

$$PC^+ \text{ cytochrome } f \underset{k_3}{\overset{k_2}{\rightleftharpoons}} PC \text{ cytochrome } f^+$$

which leads to a concentration $[PC^+]$ equal to 25 %, 200 μs after the flash. Thus, the different equilibrium constants between PC and cytochrome *f* have been tested in order to fit the experimental curves. The best approach to the experimental curves corresponds to an equilibrium constant equal to 2 between PC and cytochrome *f* and to the rate constant: $k_2 = 14 \cdot 10^3\ s^{-1}$ and $k_3 = 7 \cdot 10^3\ s^{-1}$.

This equilibrium constant contradicts the redox potentials measured on chloroplasts, in the darkness, by Malkin et al. [9].

The similar half-times of P^+-700 reduction and cytochrome *f* oxidation reveal that transfer from cytochrome *f* to PC^+ has a greater rate constant than transfer from PC to P^+-700.

In this paper, we have mainly studied the mechanisms of oxidation of PC and cytochrome *f*. The following study will be centered around the mechanisms of reduction.

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