EVIDENCE FOR A NEW ELECTRON DONOR TO P-700 IN *CHLORELLA PYRENOIDOSA*

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1. Introduction

The oxidized photoactive chlorophyll of system I (P^+-700) formed by a laser flash in spinach chloroplasts is reduced in two phases [1,2]: one with ~10 μs half-time; the second with ~100 μs half-time. The presence of a rapid reduction phase was confirmed by measuring the turnover of system I [3] and by spectroscopic measurements in the infrared region [4,5]. This rapid reduction was also observed in blue-green algae [6] and recently in *Chlorella* [7].

The nature of the electron donor responsible for the rapid reduction of P^+-700 is still an open question. Haehnel [8] has proposed that it is cytochrome *f*. However, the oxidation rates of cytochrome *f* that he has reported ($\leqslant$ 40 μs) contradict other oxidation kinetics [9,10]: this discrepancy may be due to the fact that his measurements are not corrected for C-550 [11].

The rapid electron donor could also possibly be plastocyanin. However, the oxidation kinetics of plastocyanin [8,10] after a flash seem slower than the rapid phase of reduction of P^+-700. Some error may be attached to the kinetics of PC^+ at short times [10] since the contribution of ferredoxin–NADP-reductase [12] was not subtracted.

We have therefore reinvestigated this problem using spectroscopic measurements from 400–580 nm. Since it has been shown that the reduction kinetics of P^+-700 after a flash are highly dependent on the number of photoreactions occurring per system I center during the flash [2,7], we took this dependence into account.

Abbreviations: FNR, ferredoxin-NADP-reductase; DCMU, 3(3,4-dichlorophenyl)-1, 1-dimethylurea; PC, plastocyanin; cyt. *f*, cytochrome *f*

2. Materials and methods

Chlorella pyrenoidosa was grown in Knop medium with Arnon's trace elements A_5 and B_6. Before use, the cells were resuspended in 0.1 M phosphate buffer (pH 7) containing 7% Ficoll. Photosystem II was blocked by preillumination in the presence of hydroxylamine (10^{-4} M) and DCMU (10^{-5} M) [13]. Cells were dark-adapted for 10 min before introducing them into the cuvette for a measurement.

The absorption changes were measured using the flash detector differential spectrophotometer in [14] and modified to increase its sensitivity by P. Joliot with the collaboration of D. Béal and B. Frilley. Red filters (Wratten 29) were placed in front of the actinic flashes, and complementary filters (Schott BG 38 + Wratten 34 from 400–450 nm, + Wratten 44A from 450–550 nm, + Wratten 55 from 550–560 nm, + Schott VG 3 from 560–580 nm) in front of the photocells.

The concentration of chlorophyll (10 μg/ml) was within a range where the $\Delta A_{400-580\ nm}$ depended linearly on the concentration of algae. The absorption changes reported were the average of the absorption changes of 100 actinic flashes, with a new sample into the cuvette before each flash. Under these conditions, the changes $\Delta I/I$ were measured with a margin of error of $\pm 5 \times 10^{-6}$.

We have chosen xenon actinic flashes General Radio (Stroboslave), because of their high reproducibility. The flashes were used with their medium capacity, to decrease the probability of double photoreactions during the flash. Half of the light energy was distributed in 4 μs (instead of 7 μs with the high capacity used in [7,10]).

The average number of photoreactions occurring

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per reaction center of system I during the xenon flash was measured by the electrochromic effect drawn from the difference [15]:

$$\frac{\Delta I}{I}\ 515\ \text{nm} - \frac{\Delta I}{I}\ 530\ \text{nm}$$

If one assumes that exactly one photochemical reaction occurs per system I center during a short saturating laser flash (duration 500 ns), the ratio between the electrochromic effect generated by a xenon flash and the electrochromic effect generated by the laser flash is the mean number of photochemical reactions per system I center induced by the xenon flash. Using this method, we determined that the flash used in [10] and the flash used in this work induced, respectively, 1.9 and 1.3 photochemical reactions per system I center (fig.1).

At short times after the flash, an absorption change due to the formation of a triplet state of carotenoids is detected [16,17]. Its contribution is eliminated as described in [18]. At every wavelength, an electrochromic effect interferes with the absorption changes due to the electron donors and acceptors. It is possible to obtain the spectrum and the kinetics of the electrochromic effect [15] and thus to compute the electrochromic effect at every time and at every wavelength, and to subtract it from any measurement.

In order to make possible the comparison of the absorption changes measured in *Chlorella* with the absorption changes (spectra and extinction coefficients) observed for molecules in solution or in particles, in addition, we corrected for the 'particle flattening effect' [19] according to the method developed in [20]. Figure 1 in [15] presents the differential flattening factor of *Chlorella*, i.e., the ratio between the absorption change in solution and the absorption change in cells, as a function of the wavelength.

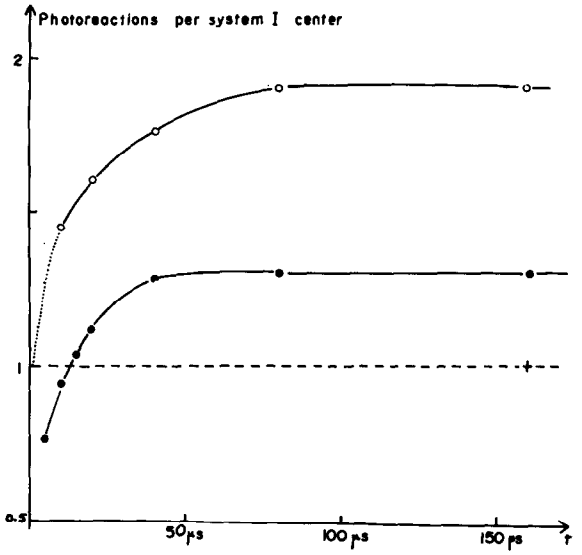

Fig.1. Average number of photochemical reactions per system I center induced by a flash as a function of the time after the beginning of the flash. (○) Xenon flash Stroboslave with high capacity; (○) Xenon flash Stroboslave with medium capacity; (+) Saturating dye laser flash.

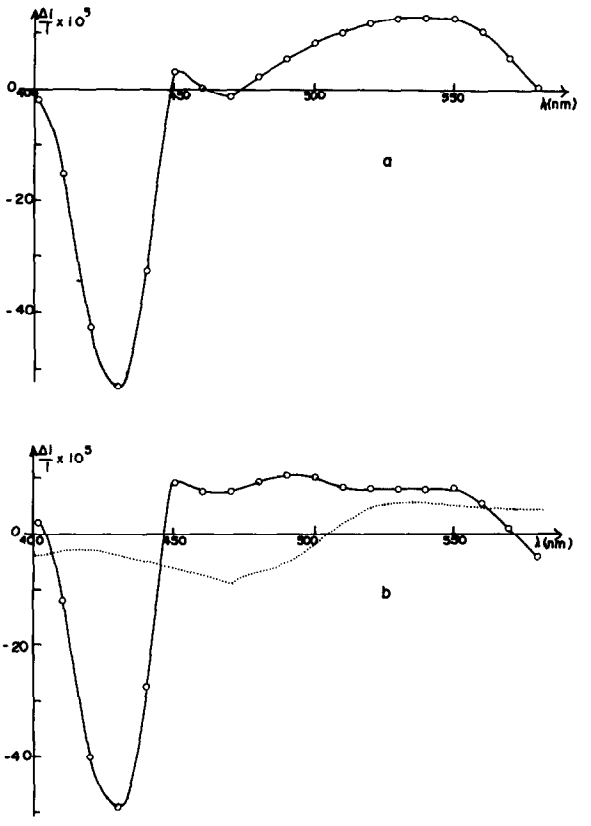

Fig.2a. Absorption changes 2 μs after a flash (after subtraction of the carotenoid triplet change and of the electrochromic effect) as a function of the wavelength. 2b. Dotted line: contribution of FNR. The spectrum of FNR–FNR in whole *Chlorella* cells is drawn from [15]. Open circles: same as 2a, but after additional subtraction of the contribution of FNR.

3. Results and discussion

3.1. *Spectrum of P^+-700 in* Chlorella

Figure 2a presents the absorption changes observed 2 μs after the peak of a non-saturating flash, after correction for the electrochromic effect and for the changes due to the carotenoid triplet. The peak at 430 nm is characteristic of P^+-700 [21]. The minimum at 470 nm and the maximum at 530 nm indicate the presence of some half-reduced ferredoxin–NADP-reductase $\dot{F}NR$ [12,22]. Since we already know the spectrum of ($\dot{F}NR$–FNR) in *Chlorella* [15], if we assume that the increase of absorption change from 470–530 nm is mainly due to $\dot{F}NR$, we can subtract the contribution of $\dot{F}NR$ (fig.2b, dotted line) from the absorption change reported in fig.2a. The remaining change (fig.2b, open circles), when corrected for the 'particle flattening effect' (fig.3), is very close to the spectrum of P^+-700–P-700) in spinach and *Anabaena* particles [21]. Thus it is reasonable to assume that the spectrum reported in fig.2b (open circles) is the spectrum of P^+-700–P-700) in whole *Chlorella* cells. A slight broadening of the band at 430 nm is due to the bandwidth (around 50 Å) of the interference filters that we used for detecting the flash.

This spectrum shows that the difference:

$$\frac{\Delta I}{I}\ 450\ \text{nm} - \frac{\Delta I}{I}\ 430\ \text{nm}$$

can be used as a relative measurement of the amount of P^+-700 (the changes due to $\dot{F}NR$ and $\dot{F}NR$ [22] are negligible in this quantity). Thus the kinetics of P^+-700 can be measured by the variation of this difference as a function of time. The kinetics of the oxidized plastocyanin (PC^+) may be obtained at the isobestic point (572 nm) of the transition P-700→P^+-700 after correction of a small contribution due to $\dot{F}NR$; $\dot{F}NR$ can be drawn as indicated in [15] by the difference:

$$\frac{\Delta I}{I}\ 530\ \text{nm} - \frac{\Delta I}{I}\ 506\ \text{nm}$$

The kinetics of oxidation cyt. f^+ are obtained by the difference [10]:

$$\frac{\Delta I}{I}\ 545\ \text{nm} - \frac{\Delta I}{I}\ 553\ \text{nm}$$

the blockage of system II avoids any contribution from C-550).

3.2. *Evidence for a new electron donor to P^+-700*

Figure 4 presents the kinetics of P^+-700, PC^+ and cyt. f^+ after a flash. 5 μs after the beginning of the flash, an average value of 0.75 photochemical reactions per system I center occurred (fig.1), and thus at this time the proportion of oxidized P-700 is $\leqslant 75\%$. From this estimate and from the kinetics of fig.4a, we conclude that the proportion of oxidized P-700 is $\leqslant 55\%$, 10 μs after the beginning of the flash, when an average value of 0.95 charge separations per system I center occurred. Despite this, almost no oxidation of either PC or cyt. f is observed at this time. The difference spectrum drawn 15 μs after the flash (fig.5a, open circles) confirms that at this time, no PC^+ nor cyt. f^+ are observed. We can deconvolute this spectrum into the sum of the spectra of (P^+-700–P-700) and ($\dot{F}NR$–FNR) (fig.5b) and still remain within the margin of error (fig.5a, dotted lines).

Thus we must admit that the phase of reduction

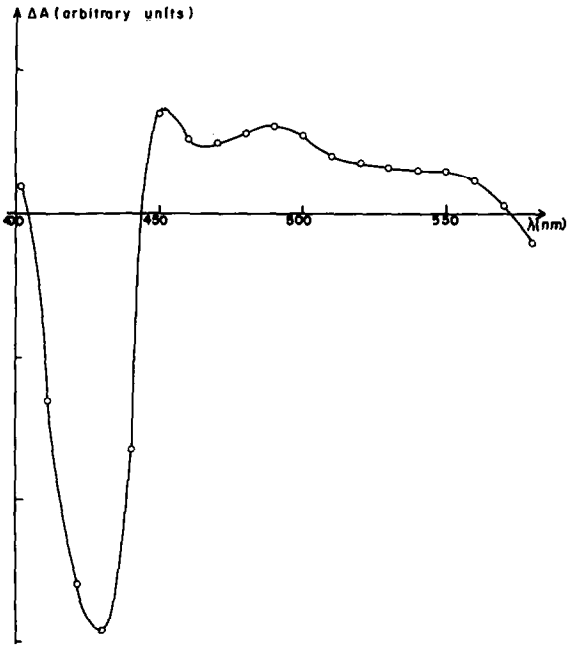

Fig.3. Spectrum presented on fig.2b (open circles) corrected from particle flattening effect.

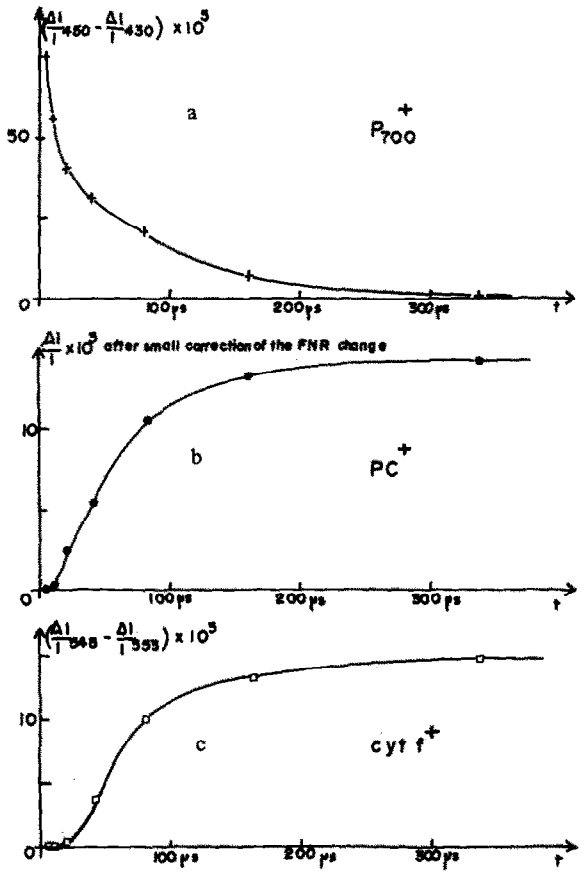

Fig.4. Differences of absorption changes as a function of the time after a flash (after subtraction of the carotenoid triplet change and of the electrochromic effect):

(a) $\Delta I/I$ 450 nm–$\Delta I/I$ 430 nm.

(b) $\Delta I/I$ 572 nm after subtraction of a small change due to FNR

(c) $\Delta I/I$ 545 nm–$\Delta I/I$ 553 nm

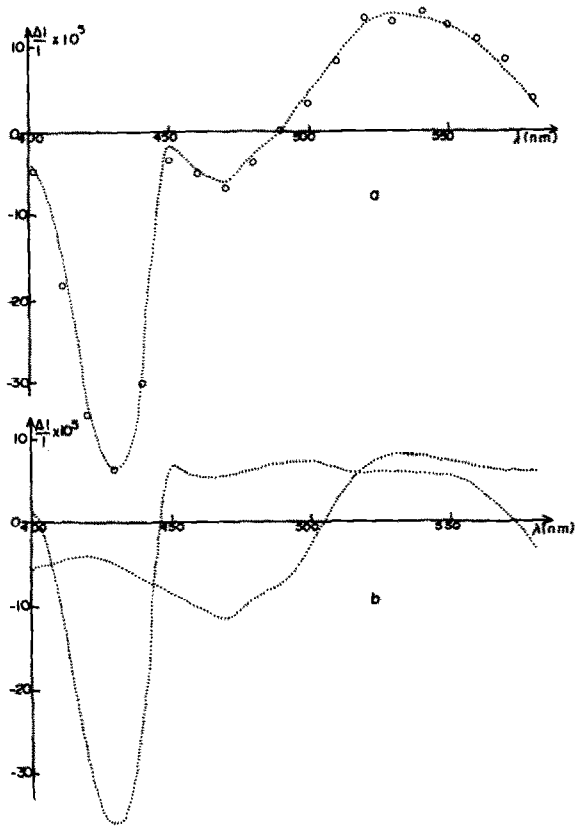

Fig.5a. Open circles: absorption changes 15 μs after a flash (after subtraction of the carotenoid triplet change and of the electrochromic effect) as a function of the wavelength. Dotted line: sum of the absorption changes presented in fig.4b. 5b. Contributions of FNR (the spectrum of FNR–FNR in whole *Chlorella* cells is drawn from [15].and of P-700 (the spectrum P^+-700–P-700 in whole *Chlorella* cells is drawn from fig.2b).

of P^+-700 which takes place in the 10 μs range is associated with the oxidation of an unknown electron donor that we shall name PD (primary donor to P-700) according to the terminology in [1]. The present work demonstrates that PD is neither PC nor cyt. *f*, and that the eventual absorption changes of (PD^+–PD) are inferior to the uncertainties of our measurements between 400 nm and 580 nm.

The margin of error, in the measurement of an absorption change, $\Delta I/I$, corrected for the carotenoid triplet and the electrochromic effect, is 10^{-5}. The kinetics calculated from the difference of the absorption changes at two wavelengths thus present an error margin of 2×10^{-5}. With these uncertainties, the lag observed in the oxidation of PC (fig.4b) is not significant. However, the margin of error can be decreased below 10^{-5} by drawing complete spectra. Thus, we observe that 15 μs after the flash the absorption change due to PC^+ at 580 nm is within the error margin of 10^{-5} (fig.5a) and that 40 μs after the flash, it is around 5×10^{-5} (not depicted on figure): the oxidation of PC^+ indeed presents a lag during about 15 μs.

This result argues in favour of a series scheme:

Cyt. *f*–PC–PD–P-700

This scheme is also consistent with [23]: in the absence of the subunit III of the reaction center I, the electron transfer from plastocyanin to P^+-700 is inhibited. Most likely PD is included in the subunit III of the reaction center I.

Acknowledgements

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