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TURNOVER KINETICS OF PHOTOSYSTEM I MEASURED BY THE ELECTROCHROMIC EFFECT IN *CHLORELLA*

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Summary

The rise kinetics of the absorption changes induced at 515 nm and 480 nm by a flash were studied using two types of xenon flashes of different durations. The 'slow' rise of the absorption change ($t_{1/2}$ = 15—20 μs) observed by Cox and Delosme (1978 C.R. Acad. Sci. (Paris) Sér. D 282, 775—778) and Joliot P., Delosme, R. and Joliot, A. ((1977) Biochim. Biophys. Acta 459, 47—57) was found to be due to double hits occurring in the reaction centers of System I during the flash.

The turnover kinetics of the reaction centers of System I after a short flash were studied by a double flash method. They are in agreement with a second order reaction between P^+-700 and its electron donor.

Introduction

The rise time of the electrical potential difference generated across the thylakoid membrane of green plants by flash illumination was estimated by Wolff et al. [1]. Using a repetitive laser actinic flash lasting 25 ns at half-height, they measured the risetime of the absorption increase at 515 nm induced by the flash, under conditions where this absorption increase is proportional to the increase of electrical potential (electrochromic effect). They concluded that the half-rise time was shorter than 20 ns in spinach chloroplasts.

In contrast to this finding of Wolff et al., Cox and Delosme [2] and Joliot et al. [3], using xenon actinic flashes lasting a few microseconds, observed that the fast rise of the electrochemic effect was followed by a 'slow' rise in the time range of 4—100 μs, with a half-rise time of 15—20 μs. For a saturating flash, the slow rise was induced by Photoreaction I only; Photoreaction II

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea.

induced only the fast rise (<4 μs) at 480 nm and 515 nm. As it was thought that the duration of the flash was very short compared to the turnover time of the reaction centers of System I in *Chlorella* ($t_{1/2} \simeq$ 100 μs according to ref. 4), the authors assumed that during a saturating flash, the efficiency of the photons distributed in the tail of the flash was negligible. We again studied this question by measuring more precisely the turnover time of the reaction centers of System I, and the effect of the tail of the flash on the efficiency of Photoreaction I in *Chlorella*.

Materials and Methods

The differential spectrophotometric method that we used has been described in previous papers [4,3]. For the present work, we used two types of actinic xenon flashes, General Radio, Stroboslave type 1539 A ('long' flash used in all the previous experiments described in refs. 2—4), and Verrerie Scientifique, CA 972 ('short' flash).

Fig. 1 shows the time distribution of the actinic light during the flash. It can be seen that this distribution is strongly dependent on the wavelength of emission, in the case of the long flash, half of the total energy is distributed during the first 2.5 μs for blue light ($\lambda <$ 490 nm), but 7 μs for red light ($\lambda >$ 600 nm). Thus the red actinic flash used in refs. 2 and 3 had a significant 'tail' in the 50 μs range, although the duration at half height was only 3.3 μs. For the second type of xenon flash used in the present work (CA 972), half of the total energy in red light ($\lambda >$ 580 nm) is distributed in the first 1.6 μs. The duration at half height is about 1 μs.

All the following experiments were performed at 20°C using *Chlorella pyrenoidosa* suspended in 100 mM phosphate buffer (pH 7) plus 7% Ficoll. The concentration of chlorophyll varied from 10 to 25 μg/ml, depending on the experiments.

As the subject of this study was the risetime of the electrochromic effect (absorption changes due to the generation of electrical potential), we sub-

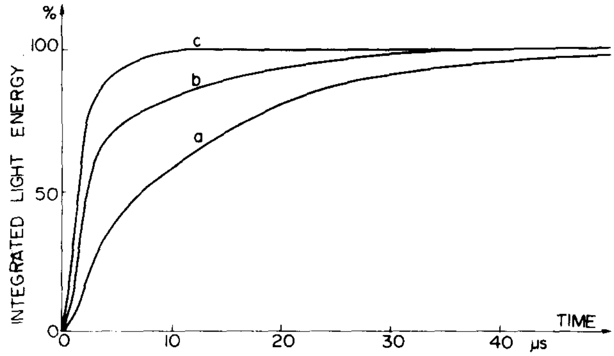


Fig. 1. Time distribution of the actinic light during (a) a long red flash (filter Wratten 29, $\lambda >$ 600 nm); (b) a long blue flash (filters Schott BG38 plus Wratten 34, $\lambda <$ 490 nm); (c) a short red flash (filter Wratten 24, $\lambda >$ 580 nm).

tracted the absorption change due to carotenoid triplet states from our measurements, according to the method described in ref. 3. Photoreaction II was blocked by the addition of DCMU and hydroxylamine. To measure the absorption change due to the carotenoid triplet state, we blocked Photoreaction I with continuous light strong enough to keep the reaction centers in the oxidized state: thus no electrical potential was generated by the flash; the absorption change induced by the flash under these conditions was subtracted from the measurements made without continuous light. In the experiment shown in Fig. 4, however, this correction was not applied, because the formation of carotenoid triplet is negligible when System II is not excited (far red actinic light) [3]. In the same way no correction was made for the experiment of Fig. 5, because the formation of carotenoid triplet gives no significant absorption change at 480 nm [2].

Results

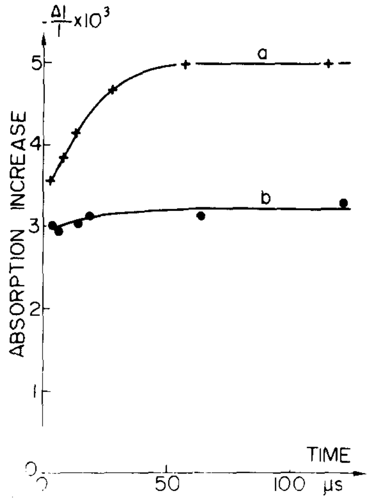
In Fig. 2, we compared the absorption changes at 515 nm due to Photoreaction I induced by the two types of strong actinic flash.

(a) The long flash gave rise to a biphasic change, with a large slow component (magnitude = 1/3rd of the total change, and $t_{1/2} \simeq 15$ μs).

(b) On the contrary, the short flash induced practically only the rapid change, followed by a very small slow phase (7% of the total change). Thus the magnitude of the slow rise increases with the duration of the actinic flash. This result suggests that a second photoreaction occurs during the long flash because of the absorption of two successive quanta in the same reaction center of System I. This implies that after the first photoreaction, at least part of the reaction centers of System I are reactivated in a few microseconds. Although its energy is nearly saturating, the short flash (curve b) gives a much lower probability of efficient double hits in the reaction centers of System I than the long flash. However, we cannot exclude that a significant amount of double hits occurs during the 'rapid' phase (<4 μs) of curve b.

Fig. 3 shows the saturation curves of the absorption change at 515 nm induced by (a) a long flash, and (b) a short flash. The measuring flash was given 116 μs after the actinic flash, i.e. a long time compared to the total duration of the actinic flash. For a very weak actinic flash (i.e. when the probability of double hits during the flash is negligible), the absorption change measured 116 μs after the flash must be independent of the duration of the flash. Thus the two saturation curves of Fig. 2 were normalized to the same slope at zero intensity. If the absorption increase was due to a single photoreaction of System I in the whole range of intensities, we would expect to find the same saturation curve for both types of actinic flashes, independent of the time distribution of the actinic light. On the contrary we observe that the two saturation curves are significantly different. The saturation requires more energy for the long flash, which confirms that in this case more than one photoreaction per center occurs during the flash.

In Fig. 4 we measured the kinetics of the absorption change at 515 nm and 480 nm following a short flash of low intensity (far red light). In this case we observe no significant slow absorption change between 10 and 160 μs. Thus the



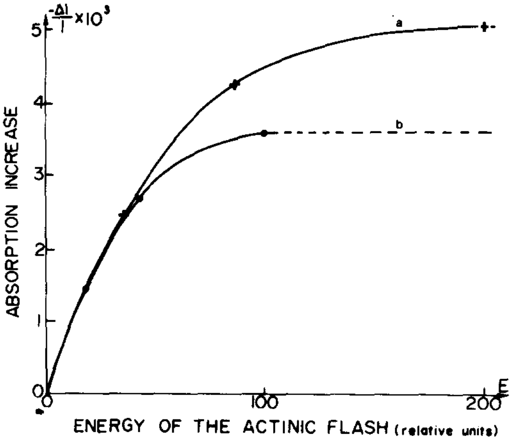
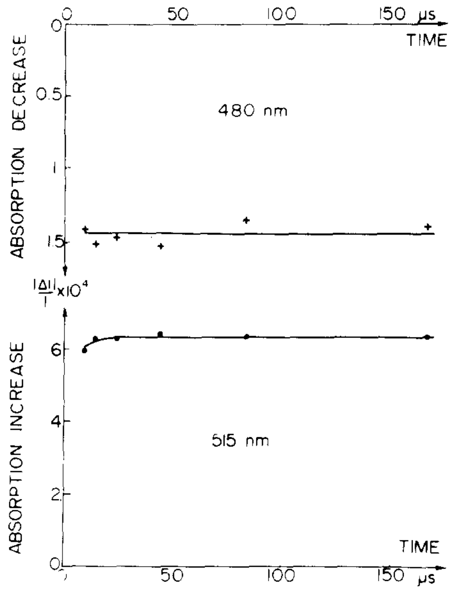


Fig. 2. Time course of the absorption change at 515 nm due to Photoreaction I (a) after a long saturating flash; (b) after a short saturating flash. Red actinic light (filter Wratten 24, λ > 580 nm). *Chlorella* + 20 µM DCMU + 100 µM hydroxylamine.

Fig. 3. Absorption change at 515 nm due to Photoreaction I, measured 116 µs after (a) a long flash; (b) a short flash, as a function of the energy *E* of the flash. Red actinic light. *Chlorella* + 20 µM DCMU + 100 µM hydroxylamine.



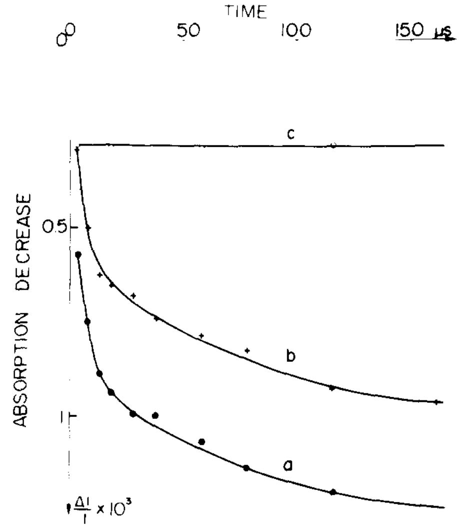


Fig. 4. Time course of the absorption changes at 480 nm and 515 nm following a short flash of low energy. Far red actinic light (filters Wratten 24 plus 97, λ > 690 nm). *Chlorella* wihout addition of inhibitor.

Fig. 5. Time course of the absorption change at 480 nm following a long saturating flash of red light. (a) Without inhibitor (Photoreactions I + II); (b) 20 µM DCMU plus 200 µM hydroxylamine (Photoreaction I alone); (c) difference (a — b) (Photoreaction II alone).

'slow' component of the absorption change observed in the 100 μs range in refs. 2 and 3 was due to the tail of the long actinic flash used.

Fig. 5 shows the kinetics of the absorption change at 480 nm following a long flash of saturating intensity, (a) when both photosystems are active, and (b) when only Photosystem I is active. The two curves are parallel, and the difference between them (curve c) represents the change due to Photoreaction II. Thus Photoreaction II induces no slow absorption change due to double hits occurring during the tail of the long flash. We observe that the slow change at 480 nm due to Photoreaction I is larger than at 515 nm (70% of the total change), and that it is biphasic. Thus the slow change at 480 nm is likely to include at least two distinct components. The first one is due to the rise of electrical potential generated by the second photoreaction (double hits), and corresponds to the component observed at 515 nm. A second absorption change ($t_{1/2} \approx 60\ \mu$s) of unknown origin could be due to some redox changes in the electron carriers involved in this photoreaction.

In the experiment of Fig. 6 we used the ability to generate electrochromic changes at 515 nm as a monitor of the activity of the reaction centers of System I (see ref. 4). This figure shows the kinetics of reactivation of the System I reaction centers following an actinic flash. The activity of the reaction centers was measured by a second actinic flash given at a variable time after the first one, and followed at 109 μs by a measuring flash of 515 nm. The absorption change induced by two synchronized actinic flashes was subtracted from the measurements. Using this method, even in the case of non saturating flashes, we take into account only the centers which have been hit a first time during the first flash and react anew during the second flash. To facilitate the comparison between the different curves, we normalized them to the same maximum amplitude. In fact the measured variation (and consequently the precision of the measurement) decreases strongly with the energy of the flash: the maximum variation was $\Delta I/I = 27 \cdot 10^{-4}$ for curve a, $18 \cdot 10^{-4}$ for curve b,

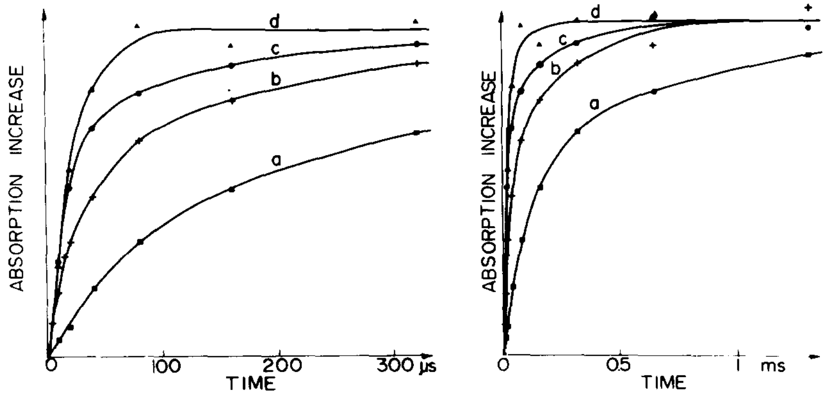


Fig. 6. Reactivation of the reaction centers of System I following (a) a long saturating flash; (b) a short saturating flash of high energy (E = 100); (c) a short flash of medium energy (E = 43); (d) a short flash of low energy (E = 18). Red actinic light. *Chlorella* + 20 μM DCMU + 100 μM hydroxylamine. The maximum variation was $\Delta I/I = 27 \cdot 10^{-4}$ for curve a, $18 \cdot 10^{-4}$ for curve b, $8 \cdot 10^{-4}$ for curve c and $3 \cdot 10^{-4}$ for curve d.

$8 \cdot 10^{-4}$ for curve c and $3 \cdot 10^{-4}$ for curve d. On this figure, we observe that (a) after a long saturating flash (curve a) the reactivation of System I is slow, with a half-turnover time of about 150 μs (cf. 4); (b) after a short flash of high intensity (curve b) the reactivation is much faster, with a half-time of 40 μs, but the shape of the curve remains non-exponential; (c) when we decrease the intensity of the short flash (curves c and d) the half-time decreases to 15—20 μs; the curve tends to become monophasic (curve d) and approximates an exponential function, within experimental error. In this experiment the signal generated by the two synchronized actinic flashes includes a certain number of double hits difficult to evaluate. Thus in Fig. 6 the amplitude of the fast phase of reactivation is probably underestimated, and the limit value of 15—20 μs for the half reactivation time is likely overestimated. The actual half-reactivation time is probably shorter.

Discussion

Our first conclusion is that no slow rise of the electrical potential difference across the thylakoid membrane occurs in the 3—100 μs range following a short flash which induces a single turnover of the reaction centers. This result is consistent with those of Wolff et al. [1] and rules out the interpretations given in ref. 3.

Secondly, the present work suggests that in *Chlorella* at least some of the reaction centers of System I are reactivated rapidly (half-turnover time = 15—20 μs) after a first photoreaction. Thus an electron donor and an electron acceptor are able to react rapidly with a fraction of the primary oxidant (P^+-700) and reductant (X^-) generated by the short flash. As the half-turnover time of the primary electron acceptor X of System I is very likely less than 3 μs (refs. 5 and 6), the fast turnover that we observe is probably that of a fraction of the primary donor *P*-700.

Little information exists about the turnover kinetics of *P*-700 in *Chlorella*. Joliot and Delosme [4] and Joliot and Joliot [7] made direct measurements of the turnover of the System I reaction centers. They measured the recovery of the ability to generate electrochromic effect at 520 nm after a xenon flash of a few microseconds. They found non-exponential kinetics with an overall half-turnover time of 100—200 μs. Hiyama and Ke [8] studied the reduction of P^+-700 in the blue-green alga *Plectonema*, by measuring the absorption changes at 435 nm following a laser flash of approx. 20 ns. They observed two phases of reduction ($t_{1/2} \approx 10$ μs and 200 μs).

In spinach chloroplasts, the turnover-time of *P*-700 has been estimated by different authors. Haehnel et al. [9,10] studied the reduction of P^+-700 by measuring the absorption changes at 703 nm following a nearly saturating flash of 0.5 μs. They found polyphasic reduction kinetics, that they resolved in two first order reactions with half-reaction times of about 10—20 μs and 200 μs. They suggested that these two reactions involved two electron donors, cytochrome *f* and plastocyanin.

Warden and Bolton [11] measured the decay of the electron spin resonance signal I following a saturating flash in spinach chloroplasts, as an indicator of the reduction of P^+-700. They found that the decay was strongly dependent on the duration of the flash. They concluded that (a) following a laser flash of about 0.2 μs, P^+-700 was probably reduced within 10 μs by a primary electron donor; (b) during a flash of 10 μs, about 50% of signal I was reduced within a few microseconds, and some centers reacted twice during the flash.

Bouges-Bocquet [12] measured the kinetics of reactivation of System I in spinach chloroplasts after a flash. Using a double flash method similar to our experiment of Fig. 6, she measured the ability of the chloroplasts to reduce methylviologen. She found that the reactivation kinetics during the first 500 μs were not exponential, but she ascribed the shape of the time curve to a second order reaction

$$\mathrm{PD} + P^+ \rightarrow P + \mathrm{PD}^+ \quad (\mathrm{PD} = \text{primary donor})$$

rather than two first order reductions with two different electron donors as suggested by Haehnel et al. [10].

Using differential absorption spectrophotometry at 823 nm, Mathis et al. [13,14] recently measured the rate of reduction of P^+-700 in spinach chloroplasts and *Chlorella* after a saturating ruby laser flash of 8 ns. In chloroplasts, half of the initial amount of P^+-700 decreased in about 100 μs with $t_{1/2}$ = 10—20 μs.

Thus various authors using independent methods of measurement concluded that the kinetics of the reduction of P^+-700 in spinach chloroplasts were not first order, and that a fraction of this component was reduced with a half-time of about 20 μs. The present work suggests that this conclusion also applies to *Chlorella*. In ref. 4 and Fig. 6 curve a, no fast turnover of System I was observed in *Chlorella* because of the long duration and high intensity of the actinic flash, which favoured a large amount of double hits during the flash; in this case a secondary electron donor was involved in the reduction of P^+-700. But when a short weak flash is used (curve d of Fig. 6), a fast turnover is observed.

A first possible explanation of a non-first order reduction of P^+-700 was suggested by Haehnel et al. [10]. They proposed that two electron donors (cytochrome *f* and plastocyanin) were connected to P^+-700, either in a linear chain:

$$\text{cytochrome } f \xrightleftharpoons{200\ \mu s} \text{plastocyanin} \xrightleftharpoons{20\ \mu s} \text{P-700}$$

or in parallel:

$$\begin{tikzcd}
\text{cytochrome} \arrow[dr, dashed, shift left, "200\ \mu s"] \arrow[dr, dashed, shift right, <-] & \\
& \text{P-700}\ . \\
\text{plastocyanin} \arrow[ur, dashed, shift left, "20\ \mu s"] \arrow[ur, dashed, shift right, <-] &
\end{tikzcd}$$

But they favoured the parallel scheme on the basis of calculations of equilibrium constants. In subsequent papers [15,17], Haehnel opted for a parallel scheme in which most of the electrons are transferred via plastocyanin. To avoid the controversy that still exists concerning the kinetic relations between cytochrome *f*, plastocyanin and *P*-700 [17,18], we refer to the electron donor

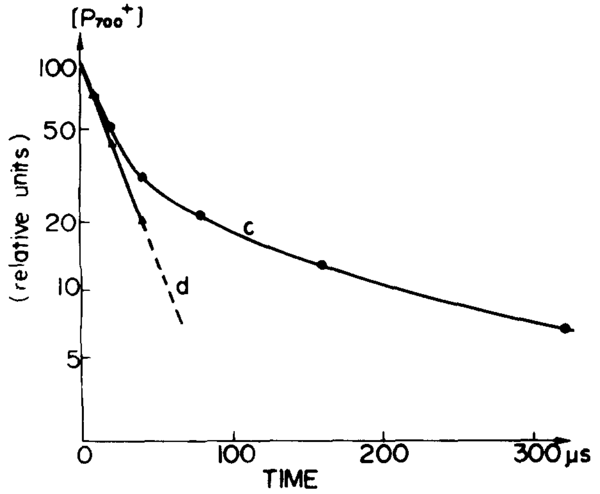


Fig. 7. Time course of the reduction of P^{+}-700 calculated from the experiment of Fig. 6. Logarithmic scale. Curves c, d correspond to curves c, d of Fig. 6. The ordinate represents the concentration of inactive centers of System I, assuming that the number of double hits during the flash is negligible.

to P-700 by the letter D. If two donors were arranged in series as follows:

$$D_2 \xrightarrow{150\ \mu s} D_1 \xrightarrow{<20\ \mu s} P\text{-}700\,,$$

a short saturating flash should be followed by a complete first order reduction of P^{+}-700 with a half-time shorter than 20 μs. On the contrary, we observe that the recovery of P-700 is not first order (Fig. 6 curves b and c, Fig. 7 curve c). In addition, Haehnel et al. [9,10] and Mathis et al. [13,14] observed a slow component in the reduction of P^{+}-700 in chloroplasts after a short saturating flash (only half of P^{+}-700 was rapidly reduced with $t_{1/2} \approx 20\ \mu s$); this is not consistent with the series model.

In the same way, these results are inconsistent with a parallel scheme

$$\begin{tikzcd} D_1 \arrow[dr, "20\ \mu s"] & \\ & P\,. \\ D_2 \arrow[ur, "150\ \mu s"'] & \end{tikzcd}$$

An alternative assumption would be that some molecules of P-700 are connected to D_1 and the other ones to D_2:

$$D_1 \xrightarrow{<20\ \mu s} P$$

$$D_2 \xrightarrow{150\ \mu s} P$$

In this case, both fast and slow reduction of P^{+}-700 would always be observed following a short flash, independent of the energy of the flash. On the contrary, we observe (Fig. 6b, c, d) that the slow turnover tends to disappear when the energy of the short flash is decreased.

In conclusion, any model in which electron donors rigidly linked to the molecules of P-700 react according to a first order scheme:

$$\mathrm{D}P^+ \rightarrow \mathrm{D}^+P$$

is inconsistent with our results. On the contrary, the results of Fig. 6 can be explained by a reaction involving co-operation between several electron transport chains, according to a bimolecular scheme:

$$\mathrm{D} + P^+\text{-}700 \rightarrow \mathrm{D}^+ + P\text{-}700\ .$$

When the energy of the short flash decreases (curve d), the amount of P^+-700 produced by the flash decreases, and the reduction of P^+-700 tends to become first order with respect to the concentration of P^+-700 (Fig. 7 curve d).

In the preceding discussion we supposed that the electron transfer from D to P was much faster than the back reaction, i.e. that the equilibrium constant was practically infinite. But we cannot exclude the hypothesis of a low equilibrium constant, and in this case the kinetic analysis is more complicated. But the main conclusion remains that the discrepancy between the reaction kinetics following a strong and a weak flash indicates a co-operation between electron transfer chains.

As pointed out by Bouges-Bocquet [12], a bimolecular reaction implies an exchange between molecules of either D, or P, or both. Haehnel [17] proposed that electron exchange occurs between the molecules of plastocyanin, the main electron donor to P-700.

The interpretation of curve b (Fig. 6) is not simple, because the high energy of the flash used ($E = 100$) probably favours a certain number of double hits difficult to evaluate. In this case, the number of oxidizing equivalents generated during the flash exceeds the number of initially available equivalents of electron donor D. We intend to avoid this difficulty in a new series of experiments using a laser flash (total duration $<0.5\ \mu$s). Preliminary experiments show biphasic kinetics similar to curve c of Fig. 6 for the turnover of Photosystem I following a saturating laser flash.

Our measurements of the turnover of the Photosystem I reaction centers, compared with the measurements of the reduction of P^+-700 made by different authors [9,10,13,14], show that the turnover is limited by the reduction of P^+-700, and not by the reoxidation of the reduced primary electron acceptor X^- of System I. This reoxidation is certainly very rapid ($<<5\ \mu$s); otherwise it would cause a lag in the initial part of the turnover kinetics following the flash, which is not observed in Fig. 6.

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