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PHOTOOXIDATION OF CYTOCHROME b_{559} AND THE ELECTRON DONORS IN CHLOROPLAST PHOTOSYSTEM II

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SUMMARY

The effect of light on the reaction center of Photosystem II was studied by differential absorption spectroscopy in spinach chloroplasts.

At −196 °C, continuous illumination results in a parallel reduction of C-550 and oxidation of cytochrome b_{559} high potential. With flash excitation, C-550 is reduced, but only a small fraction of cytochrome b_{559} is oxidized. The specific effect of flash illumination is suppressed if the chloroplasts are preilluminated by one flash at 0 °C.

At −50 °C, continuous illumination results in the reduction of C-550 but little oxidation of cytochrome b_{559}. However, complete oxidation is obtained if the chloroplasts have been preilluminated by one flash at 0 °C. The effect of preillumination is not observed in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

A model is discussed for the reaction center, with two electron donors, cytochrome b_{559} and Z, acting in competition. Their respective efficiency is dependent on temperature and on their states of oxidation. The specific effect of flash excitation is attributed to a two-photon reaction, possibly based on energy-trapping properties of the oxidized trap chlorophyll.

INTRODUCTION

Most of the information regarding the photosystem of green plants involved in oxygen evolution (Photosystem II) has been gained from oxygen evolution and fluorescence change measurements[1,2]. More recently, techniques of differential absorption spectroscopy have given evidence of three species participating in primary events at the Photosystem II reaction center: a molecule of chlorophyll *a* (named Chl a_{II} by Döring *et al.*[3], and P-680 by Floyd *et al.*[4]) supposed to be the photoactive trap; a pigment of unknown chemical nature named C-550 by Knaff and Arnon[5]; and a cytochrome of the *b*-type named cytochrome b_{559}, owing to the position of its α-band at room temperature[4,6–8]. In their comparative study of fluorescence induction and of C-550 absorbance changes, Erixon and Butler[9] provided good evidence for the identification of C-550 as being the primary electron acceptor in a photochemical

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

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reaction that leaves chlorophyll a_{II} (P-680) in an oxidized state. As cytochrome b_{559} is photooxidized at -196 °C, it might be a primary electron donor; Floyd *et al.*[4] were indeed able to follow the concomitant reduction of oxidized P-680 and oxidation of cytochrome b_{559}. However, room temperature experiments are ambiguous, as they do not allow one to determine whether cytochrome b_{559} is actually involved as electron donor in Photosystem II reactions of untreated chloroplasts[10–12]. Part of the ambiguity arises from the fact that cytochrome b_{559} can exist in chloroplasts under different states having different redox potentials, and is probably associated with different photoreactions at room temperature[12,13].

Using a sensitive technique of differential absorption spectroscopy, we studied the effect of illumination of spinach chloroplasts under continuous or flash light, at different temperatures. We also made use of flash preillumination, accumulating one positive hole on the oxygen side of reaction center 2 (ref. 2). Our experiments demonstrate a different effect of both types of illumination, best interpreted as some kind of two-photon effect at reaction center 2. They also indicate that one electron acceptor (C-550) is coupled with two different electron donors (one of them being cytochrome b_{559}) whose effectiveness is dependent on the state of the chloroplasts: temperature, and level of oxidation on the reducing site of reaction center 2.

MATERIAL AND METHODS

Biological material

Chloroplasts were prepared from fresh spinach leaves, by a 15-s grinding in a Waring blender, in a solution of 0.4 M sucrose–0.02 M Tris buffer (pH 8.0). After filtration through eight layers of cheese-cloth and centrifugation at 1500 × *g* for 10 min, the pellet was homogenized in a solution of 0.4 M sucrose–glycerol (1:2, by vol.), at a concentration of 100–500 μg chlorophyll per ml. Glycerol was added to prevent freezing and thermal denaturation[14]. The suspension was kept in ice for rapid use (less than 1 h); for day-long series of experiments it was kept in liquid N_2 and rewarmed just before use[15]. In experiments with 3-(3,4-dichlorophenyl-1,1-dimethylurea (DCMU), this inhibitor was added to the pool of suspension used for both sample and reference cuvettes, as 1 μl of saturated ethanol solution for 0.5 ml of suspension.

Absorption spectra

All spectra were recorded at -196 °C, with a double-beam spectrophotometer (Perkin-Elmer, Model 356), equipped with its standard low-temperature accessory. We worked with constant slits of 0.25 mm ($\Delta\lambda = 1$ nm). The two cuvettes (sample and reference; optical path 1 mm) were made from two holes, covered with lucite plates, cut in a copper plate. The copper frame was dipping in liquid N_2 contained in a partly unsilvered Dewar flask. The temperature inside the cuvette was measured with a chromel–alumel thermocouple. We did not detect any photochemical effect of the measuring beam of very low intensity. Due to our experimental procedure and to the slow response of the spectrophotometer, we were only able to detect species stable for at least 5 min. In all reported spectra, increases in absorbance of the sample cuvette give upward signals.

Procedure for illumination

After transferring the suspension to the cuvettes, the chloroplasts were dark-

adapted for 10 min at 0 °C. Thereafter they were submitted to some or all of the following treatments:

(1) Preillumination, at 0 °C, by means of one or several (separated by 0.5 s) flashes from a xenon tube (2 J; 2 μs). The cell holder was then rapidly dipped into liquid N_2 within 1 s.

(2) Illumination at −50 °C, the cuvettes being cooled by a flow of air passing through ethanol–solid CO_2. Actinic white light was provided by a 100-W tungsten–iodine lamp, and passed through a 5-cm path of water. After a 5-s illumination the cell holder was dipped into liquid N_2.

(3) Illumination at −196 °C, effected inside the Dewar flask. For continuous illumination we used the standard actinic accessory of the spectrophotometer (tungsten–iodine lamp, 100 W). Monochromatic light was obtained with interference filters (Balzers, B40), at wavelengths of 652 and 696 nm. On the sample cuvette a calibrated Eppley thermopile indicated 1.0 $mW \cdot cm^{-2}$ (652 nm) and 5.2 $mW \cdot cm^{-2}$ (696 nm). For flash excitation we used either a Stroboslave illuminator (General Radio, Model 1539 A; 0.1 J, 2 μs; operated every 10 s) or a Q-switch ruby laser (C.S.F., Model LA 632; 300 mJ of light, 50 ns; one pulse every 100 s).

The reference cuvette could be protected from stray light by two masks. In some instances it was illuminated, but only at −196 °C.

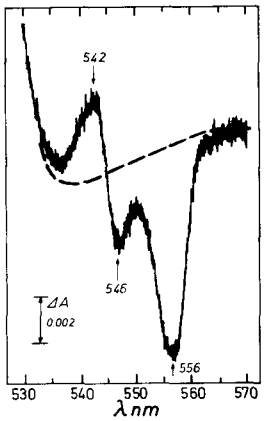
All flash illuminations reported here were saturating in the sense that decreasing the intensity by a factor of two did not change the effects. We were not able to measure the light energy received by the cuvette in flash excitation and to compare with continuous light illumination. Geometric arrangements and the percentage of collection of emitted light, in the different types of illumination, were about the same (excepted for the laser beam).

RESULTS

Illumination of chloroplasts at −196 °C

The effect of continuous illumination at −196 °C is reported in Fig. 1 as a light *minus* dark difference spectrum. This spectrum has been reported by several authors[6–9]; the negative peak at 556 nm is attributed to the photooxidation of cytochrome b_{559}, and the double peak (positive at 542 nm, negative at 546 nm) to the photoreduction of C-550. After illumination of the reference cuvette (Fig. 1), the difference spectrum is identical to a base line, by saturation of the light effects on both sides. Making use of techniques described earlier[7,13,16] we measured the difference spectra of chloroplast suspensions at −196 °C in the presence of different redox reagents in the two cuvettes (ferricyanide *minus* hydroquinone, ferricyanide *minus* ascorbate, ascorbate *minus* dithionite); we determined that in our preparations 70–80% of the cytochrome b_{559} was in the high-potential state, and that 70% of the cytochrome b_{559} high potential, reduced in the dark, could be photooxidized at −196 °C.

There is a parallel increase of both C-550 and cytochrome b_{559} signals under progressive illumination, as shown by Fig. 2. An identical difference spectrum and the same parallel increase of both signals were observed under illumination with white light (saturation in 0.3 s, Fig. 2) or with monochromatic light of wavelength 652 nm (saturation in 1 min 45 s) or 696 nm (half-saturated in 10 min). No effect of DCMU could be detected under illumination in the above conditions.



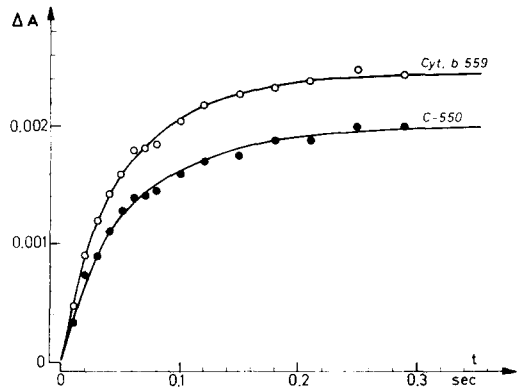


Fig. 1. Difference spectra obtained after a 1-s illumination of the sample cuvette only (—) and of both cuvettes (— —). Illuminations performed at −196 °C. Chlorophyll concentration: 350 μM.

Fig. 2. Effect of progressive illumination at −196 °C, by chopped light from a 100-W lamp. Oxidized cytochrome b_{559} (○—○) is measured as $\Delta A_{556\,nm}$, and C-550 (●—●) as ($\Delta A_{546\,nm} - \Delta A_{542nm}$). Successive difference spectra were recorded, separated by an illumination of 0.01, 0.02, 0.03 or 0.04 s (from a camera shutter). Chlorophyll concentration: 140 μM.

The effect of flash illumination is depicted on Fig. 3a, using the Stroboslave device (about 10 flashes are necessary for a complete reduction of C-550). The C-550 signal is identical to that obtained by illumination of the same chloroplasts preparation with continuous light, but there is practically no oxidation of cytochrome b_{559}. Further continuous illumination of the sample cuvette gives no change in the spectrum. The deficit of oxidized cytochrome b_{559} is made clearer by continuous illumination of the reference cuvette, which eliminates the C-550 signal and leaves oxidized cytochrome b_{559} in the difference spectrum (Fig. 3b). A similar spectrum lacking the cytochrome b_{559} signal followed excitation with the laser beam (Fig. 4a). Another common feature is that these flash excitations were unable to saturate the C-550 signal in one flash, about ten being necessary. In the experiments reported on Fig. 3 there is practically not more than 15% of the normal photooxidized cytochrome b_{559}; in all our series of experiments this percentage varied between 0 and 40%. As a general trend the lowest percentages were obtained with fresh and well dark-adapted chloroplasts. We checked that the flashes had no irreversible effect; after light saturation with flashes, the sample was re-warmed, dark-adapted, cooled to −196 °C and it displayed a regular difference spectrum upon illumination in continuous light.

The previous results hold for dark-adapted chloroplasts. With preilluminated chloroplasts, flash excitation at −196 °C results in complete oxidation of cytochrome b_{559}, as with continuous illumination (Fig. 4b). About ten saturating flashes are still necessary for a complete photochemical reaction.

Illumination of chloroplasts at −50 °C

A saturating illumination in continuous light of dark-adapted chloroplasts at −50 °C (Figs 5a and 5b) leaves all the C-550 in the reduced form, but only part of the cytochrome b_{559} is photooxidized: 10–40% of photooxidation (relative to the

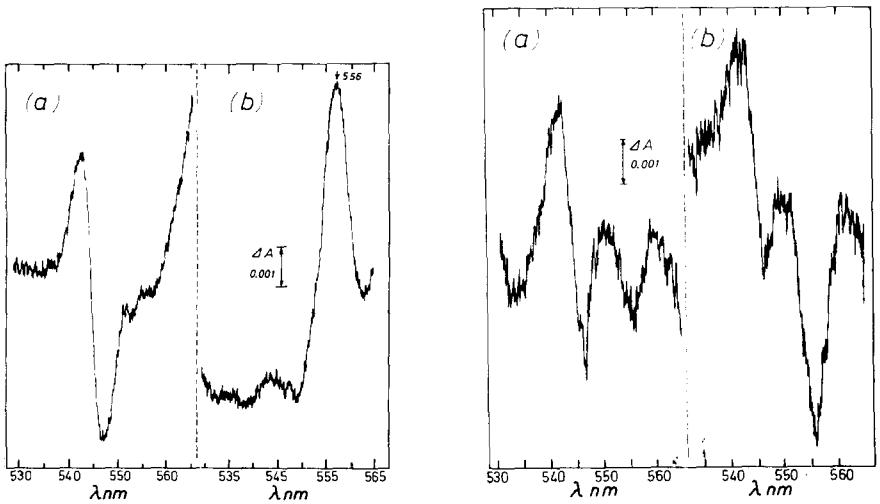


Fig. 3. (a) Difference spectrum obtained after illumination of the sample cuvette by 10 flashes (from the Stroboslave flash tube) at −196 °C. Additional illumination of the same cuvette in continuous light gave no change of the spectrum. (b) Same as (a), but after 5 s of saturating illumination, in continuous light, of the reference cuvette. Chlorophyll concentration: 350 μM.

Fig. 4. Difference spectra obtained after excitation of the sample cuvette by 4 laser flashes at −196 °C. (a) Dark-adapted chloroplasts. (b) Preilluminated chloroplasts: the sample cuvette received one flash at 0 °C before being cooled at −196 °C. The formation of C-550 is about 60% of saturation. Chlorophyll concentration: 380 μM.

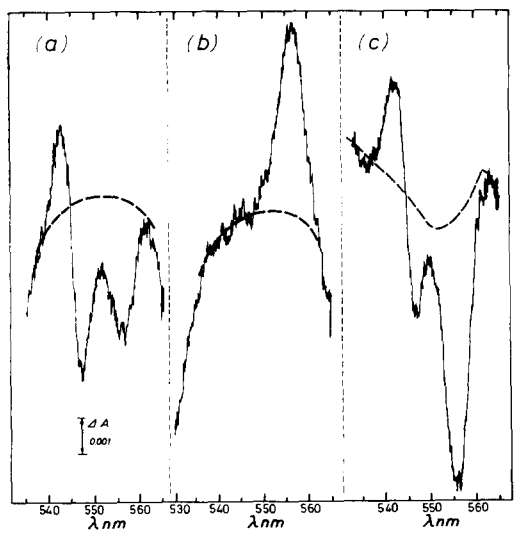


Fig. 5. (a) Difference spectrum recorded after illumination of the sample cuvette by 5 s of white light at −50 °C. — —, estimated base line. (b) Same as (a) but after saturating illumination of the reference cuvette, as Fig. 3b. — —, estimated base line. (c) —, same as (a), but with the sample cuvette preilluminated. — —, after saturating illumination of the reference cuvette, as Fig. 3b. Chlorophyll concentration: 315 μM.

amount of cytochrome b_{559} photooxidized at −196 °C), largely determined by the freshness of the chloroplasts; a smaller oxidation was obtained with fresher chloroplasts. Further illumination at −196 °C had no effect on the difference spectrum, but the peak of cytochrome b_{559} appeared after illumination of the reference cuvette (Fig. 5b). Addition of DCMU had no measurable consequence on the effect of such illumination at −50 °C (Figs 6 a and b).

Preillumination of the chloroplasts by one flash at 0 °C results in total cytochrome b_{559} oxidation by a subsequent illumination at −50 °C (Fig. 5c). However the preilluminating flash given alone has no effect on the absorption spectrum recorded at −196 °C nor on the effect of illumination at that temperature. We conclude that, if Z is the electron donor for the photoreaction at −50 °C with dark-adapted chloroplasts, this electron donor is cytochrome b_{559} with preilluminated chloroplasts. We were not able to detect any difference in the effect of preillumination by one flash or by several flashes (2–4) separated by 0.5 s.

Addition of DCMU seriously modifies the previous pattern: indeed, the illumination at −50 °C of preilluminated chloroplasts now produces no oxidation of cytochrome b_{559} (Fig. 6c). Moreover the preilluminating flash alone has a detectable effect (Fig. 6e): a part of the C-550 is left reduced, and some cytochrome *f* is oxidized (peaks at 548 and 551 nm, visible on Figs 6 c, d and e). Cytochrome *f* is not photooxidized at −196 (Fig. 1) or −50 °C (Fig. 5). The appearance of oxidized cytochrome *f* in experiments with DCMU can be rationalized if we accept that cytochrome *f* is oxidized

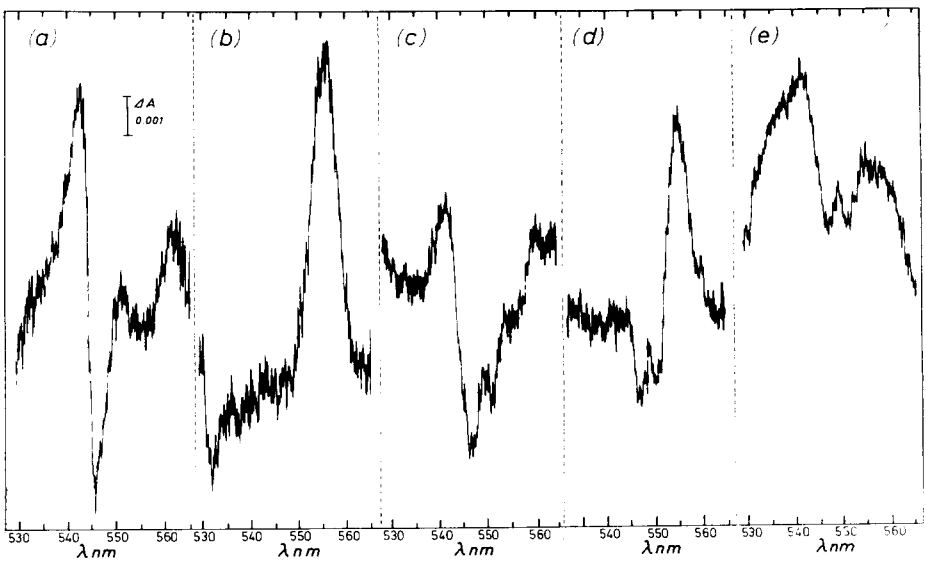


Fig. 6. Experiments with DCMU added to both cuvettes. (a) Difference spectrum recorded after illumination of the sample cuvette by 5 s of white light at −50 °C. (b) Same as (a), but after saturating illumination of the reference cuvette, as Fig. 3b. (c) Same as (a), but with the sample cuvette preilluminated. (d) Same as (c), but after saturating illumination of the reference cuvette, as Fig. 3b. (e) Control experiment for (c): after preillumination of the sample cuvette, the cell holder was dipped in liquid N_2 and the spectrum recorded. Chlorophyll concentration: 500 μM (a,b) and 315 μM (c, d, e).

by the preillumination at 0 °C, but is normally rapidly reduced by electrons coming from Photosystem II; this flow is blocked in the presence of DCMU, and cytochrome *f* is left partly oxidized.

DISCUSSION

Before discussing our results we will assume that only the Photosystem II reactions are concerned, except for the oxidation of cytochrome *f* (Fig. 6), a System I reaction[17]. This assumption is supported by previous work of several authors (see refs 5, 6, 8, 9, 18), and also by the easily interpretable influence of DCMU on the effects of illumination which we report. The scheme of Fig. 7 formally accounts for our results. Our main hypothesis is a competition between two electron donors, cytochrome b_{559} and Z (of unknown nature), at the reducing site of reaction center 2, with respective rate constants k_1 and k_2. We assume that the relative magnitude of these rate constants is temperature-dependent, with $k_1 \gg k_2$ at -196 °C and $k_1 \ll k_2$ at temperature $\geqslant -50$ °C.

The primary reaction can be considered as the step a→b. Indeed there is good evidence for the formation of the radical cation chlorophyll$^+$ as a primary reaction of reaction center 2 (refs 3 and 4). Moreover Erixon and Butler[9] found a good parallelism between the reduction of C-550 and the fluorescence quenching reaction commonly accepted as the primary reaction of Photosystem II (ref. 19). The dark reaction b→c accounts for the results of continuous illumination at -196 °C (Fig. 1) producing a stable state, characterized by reduced C-550 and oxidized cytochrome b_{559}, and not susceptible of evolution under further illumination. That reaction has been studied

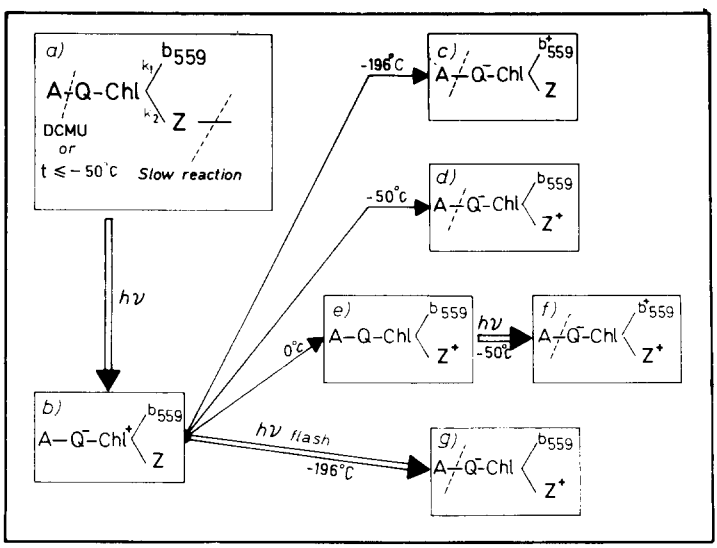


Fig. 7. Schematic representation of reactions occurring at the reaction center of Photosystem II (reaction center 2). Each block represents a state of reaction center 2. Dotted lines represent blocked or slow reactions. Double arrows are for photoreactions, simple arrows for dark steps. A is an oxidant pool[22]. The quencher Q behaves identically to C-550 (ref. 9), and b_{559} stands for cytochrome b_{559}.

by Floyd *et al.*[4], by flash spectroscopy. The authors found a half-time of 4.6 ms for the reaction, practically independent of temperature. So our hypothesis implies that temperature acts mostly on the rate constant k_2.

At temperature $\geqslant -50$ °C, cytochrome b_{559} is not the preferential electron donor as we do not detect its photooxidation at -50 °C with dark-adapted chloroplasts (Fig. 5a). Fig. 5c shows the effect of preillumination by one flash. The preilluminating short-duration flash has the effect of activating once each reaction center[2,20], and of leading to the accumulation of one oxidizing equivalent (Z^+) which can be trapped by lowering the temperature[21], when the negative charge can migrate to reduce the cytochrome *f* oxidized by the flash in a Photosystem I reaction. This leads to State e, which can be excited once only by illumination at -50 °C (ref. 22). In Fig. 5c we see that preillumination makes cytochrome b_{559} the preferential electron donor at -50 °C. So we can conclude that Z is the electron donor at -50 °C with dark-adapted chloroplasts (Step b→d).

The effect of DCMU is to block the flow of electons between Q and A (ref. 22); in these conditions reaction center 2 can turn once only and the preillumination does not lead to photooxidation of cytochrome b_{559} at -50 °C. That cytochrome *f* remains oxidized after preillumination in the presence of DCMU (Figs 6 c, d and e) is in agreement with that interpretation.

In the presence of DCMU, all the C-550 is reduced by the flash preillumination. Fig. 6c indicates that part of it has been reoxidized. This can occur by a reverse reaction (d→c), taking place before freezing at -50 °C, as indicated by experiments of delayed fluorescence and by the recovery of the fluorescence quenching capacity of Photosystem II (ref. 23).

The explanation of flash effects at -196 °C is not straightforward and requires new assumptions. The first feature is the impossibility of completely reducing C-550 by one saturating flash, about 8–10 being necessary. A similar effect has been reported by Floyd *et al.*[4] for absorbance changes in the 670–710 nm region; we do not know any experimental argument to explain this effect. The second feature is the absence of cytochrome b_{559} oxidation following flash excitation of dark-adapted chloroplasts at -196 °C, in contrast to continuous illumination. Our control experiments rule out any differential wavelength effect of the exciting light or any irreversible destruction of the reaction centers by the flashes. Possible explanations involve two-photon effects at the reaction center 2. After a first photon effecting the primary reaction a→b, we suggest that State b has quenching properties and that absorption of a second photon (before completion of the reaction b→c) will lead to some excitation of the reaction center, leading to State g. Formation of oxidized cytochrome b_{559} by flashes, after a preillumination (Fig. 4b), favors this mechanism, as Z is already oxidized. The trapping of a photon can be effected by chlorophyll$^+$, which is known to have a low energy level (12200 cm^{-1} as compared to 14700 for chlorophyll *a* (ref. 24). Another quencher could be a neighbour chlorophyll molecule if reaction center 2 contains several chlorophyll molecules as does P_{700} (ref. 25). Similar quenching properties of reaction center 2 have been inferred from low-temperature fluorescence induction curves, specially when cytochrome b_{559} is chemically oxidized[26].

The scheme of Fig. 7 is the simpler one that accounts for our results. It is probably not adequate for the inclusion of all the photochemical events occurring in the Photosystem II; more complex representations have been proposed[21], which are

not in opposition to ours. A branched scheme is not proved by our results, as a linear one (C-550–chlorophyll–cytochrome b_{559}–Z–) would be nearly as valuable. However the branched scheme seems preferable, from redox potential considerations, as it does not place cytochrome b_{559} as a necessary step for each positive hole leading to oxygen evolution: its redox potential is about +0.4 V, whereas an oxidant of potential greater than +0.82 V is necessary for oxidation of water, in a linear scheme (see refs 8, 13 and 16). Photooxidation of cytochrome b_{559} has been detected in three types of conditions: at −196 °C, at room temperature with chloroplasts whose Photosystem II is altered[6,10,12], and, as we report here, at −50 °C when at least one positive charge has been accumulated on the donor Z. This last reaction could be more relevant to the way cytochrome b_{559} actually works in normal conditions. Its role remains to be elucidated, as does the specific effect of flash excitation which we observed at low temperatures.

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