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PHOTOREDUCTION OF C-550 AND OXIDATION OF CYTOCHROME b_{559} IN CHLOROPLASTS

DEPENDENCE ON THE STATE OF PHOTOSYSTEM II

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SUMMARY

The photoreduction of C-550 and the photooxidation of cytochrome b_{559} were studied by illumination at −55 °C (by continuous white light or by 2-μs flashes) of spinach chloroplasts, which had been previously dark adapted or preilluminated at 21 °C by a variable number (n) of saturating short flashes. Differential absorption spectra were then recorded at −196 °C.

1. A saturating illumination by continuous light reduces all the C-550, but the fraction of cytochrome b_{559} which is oxidized varies with n: 32% for $n=0$; 100% for $n=2$; 36% for $n=4$. Percentages are given in respect to the amount of cytochrome b_{559} photooxidizable by continuous light at −196 °C. An oscillatory pattern is observed, which parallels the state of Photosystem II, as defined by the number of oxidizing equivalents accumulated during preillumination.

2. Excitation by a series of 10–12 saturating flashes produces the same result as continuous light. However, the effect of the first flash oscillates also with n, with a period of four. For $n=0$ (and approximately for $n=4$) 75% of the C-550 is reduced, with no oxidation of cytochrome b_{559}. For $n=2$, only about 30% of the C-550 is reduced although the flash is saturating, and some cytochrome b_{559} is concomitantly oxidized.

3. In the presence of ferricyanide which oxidizes cytochrome b_{559} in the dark, no change in the redox state of that cytochrome occurs following any illumination. For C-550, the pattern previously described is unchanged, except that not all the C-550 can be trapped in the reduced form.

The present results are discussed in relation to the properties of the reaction centre of Photosystem II, where two types of photochemical behaviour are believed to occur.

INTRODUCTION

During the last few years, two main approaches have been used in the study of Photosystem II of green plants. The first makes use of short saturating flashes,

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supposed to effect only one turnover of the reaction centres. The most striking result is the demonstration of a periodicity of four (probably related to four electrons being transferred for the evolution of one molecule of oxygen) for several observed parameters: oxygen evolution[1,2], delayed light emission[3,4] and fluorescence induction[5]. The second approach is based on absorption spectroscopy, three species being mostly studied: a special form of chlorophyll, possibly a trap, named chlorophyll a_{II} or P-680[6,7]; cytochrome b_{559}, whose role is not known and whose location in the electron transfer chain is dubious, as it can be either photooxidized[7–10] or photoreduced[11,12] in Photosystem II reactions; and C-550, chemically unknown, but is assumed to be a primary electron acceptor[13,14].

Our purpose is to establish a correlation between oscillatory properties of Photosystem II and photoreactions occurring at its reaction centre, detected by the reduction of C-550 and the oxidation of cytochrome b_{559}. Our approach is similar to that followed by Joliot and Joliot[5] who measured fluorescence induction. We previously found[15] that preillumination of chloroplasts modifies the photoreactions under flash excitation at -196 °C and under continuous illumination at -50 °C; however, we were unable to observe any oscillation. In this work, based on absorption spectra recorded at -196 °C, we do observe a periodicity of four for the properties of the photoreactions occurring at -55 °C (by continuous light or by flashes) *versus* the number of preilluminating flashes given at room temperature. The temperature of -55 °C is chosen so as to block the electron transfer from the first acceptor to the pool of secondary acceptors[16,17].

We try to interpret our results in a descriptive model of reaction centre 2, based on the electron acceptor C-550, competitive electron donors (cytochrome b_{559} and the unknown donor Z capable of releasing several electrons) and two configurations of reaction centre 2, mostly determined by the redox state of the donor Z.

MATERIAL AND METHODS

Chloroplasts were prepared as previously described[15], from young spinach leaves (8–10 weeks). They were resuspended in the grinding buffer: 0.4 M sucrose–0.02 M Tris buffer (pH 7.8)–0.01 M NaCl. The suspension was kept in ice for rapid use (less than 2 h). The chlorophyll concentration of the suspension varied from 350 to 450 $\mu g \cdot ml^{-1}$. In several experiments 10 μl of potassium ferricyanide (0.04 M) were added to 1.0 ml of suspension for both the sample and the reference cuvettes.

Procedure for illumination. Absorption spectra

The cuvettes containing the suspension of chloroplasts (optical path 1 mm) were handled as previously described[15], with a few minor modifications. The following treatments were used in succession:

Dark adaptation: 8 min at room temperature, regulated at 21 °C.

Preillumination at 21 °C, by a variable number (n) of saturating flashes, separated by 0.5 s, from a Stroboslave illuminator (General Radio, Model 1539 A, electric energy 0.1 J, duration 2 μs). The cell holder was then dipped immediately into liquid N_2, until a temperature of -70 °C was reached. It was then slowly rewarmed to -55 °C by a flow of cold air.

Illumination at -55 (± 5) °C was effected either by continuous light (a 10-s

illumination; the actinic source was a 100-W tungsten-iodine lamp) or by a variable number of saturating flashes. Two Stroboslave illuminators, exactly synchronized (jitter less than 1 μs), were used, one on either side of the sample cuvette. Two illuminators were necessary for saturation at -55 °C; a probable reason is light loss due to scattering by the microcrystallized sample. After illumination, the cell holder was rapidly dipped into a Dewar flask, containing liquid N_2 and fitted to the spectrophotometer.

Absorption spectra were recorded with a double-beam spectrophotometer (Perkin–Elmer, Model 356). After recording the difference spectrum, the sample cuvette was illuminated at -196 °C in order to check if the photoreactions were saturated. Finally the reference cuvette, protected by a mask during the preillumination at -55 °C, could be illuminated at -196 °C.

All light treatments were provided with white light. We would like to emphasize that the term "preillumination" is used for a conditioning light treatment at 21 °C (always with flashes) and "illumination" is light treatment at low temperature (with continuous light or with flashes).

RESULTS

Illumination by continuous light at -55 °C

The light *minus* dark difference spectra reveal, in the range of 530–565 nm, absorbance changes corresponding to variations of the redox state of C-550 (peaks at 542 and 546 nm), of cytochrome b_{559} (556 nm) and of cytochrome *f* (548 and 551 nm). The reduction of C-550 and the oxidation of cytochrome b_{559} (Fig. 1) are effected by the illumination at -55 °C: their difference spectra do not appear in control experiments with a variable number (n) of preilluminating flashes but no illumination at -55 °C. However, in these control experiments with an odd number of preilluminating flashes, cytochrome *f* is partly oxidized (maximum about 50%). This result might be of interest for understanding the electron transfer chain between the two photosystems and will be described elsewhere (Vermeglio, A. and Mathis, P., unpublished). The oxidation of cytochrome *f* could affect our evaluation of the amount of oxidized cytochrome b_{559}, especially after one preillumination flash (Fig. 1, $n=1$). Illumination at -55 °C did not cause any detectable oxidation of cytochrome *f*.

In all the experiments, C-550 is fully reduced following illumination by continuous light at -55 °C, but the amount of oxidized cytochrome b_{559} varies according to n. The percentages of reduced C-550 and of oxidized cytochrome b_{559} are given in respect to the amount of C-550 and of cytochrome b_{559}, respectively, which is photoreducible and photooxidizable by continuous light at -196 °C. In Fig. 1 (spectra a), further illumination of the sample cuvette at -196 °C produces no absorbance change. By saturation of the photoreactions on the reference cuvette, at -196 °C, the difference spectrum due to C-550 is cancelled, and a peak due to oxidized cytochrome b_{559} is apparent, its importance varying with n (Fig. 1, spectra b). The preillumination has no specific effect on the result of continuous illumination at -196 °C when no illumination at -55 °C is provided. The spectra of Fig. 1 correspond to the values of n giving the most contrasting results. Some distortion in the spectra is caused by uncontrolled changes in the baseline.

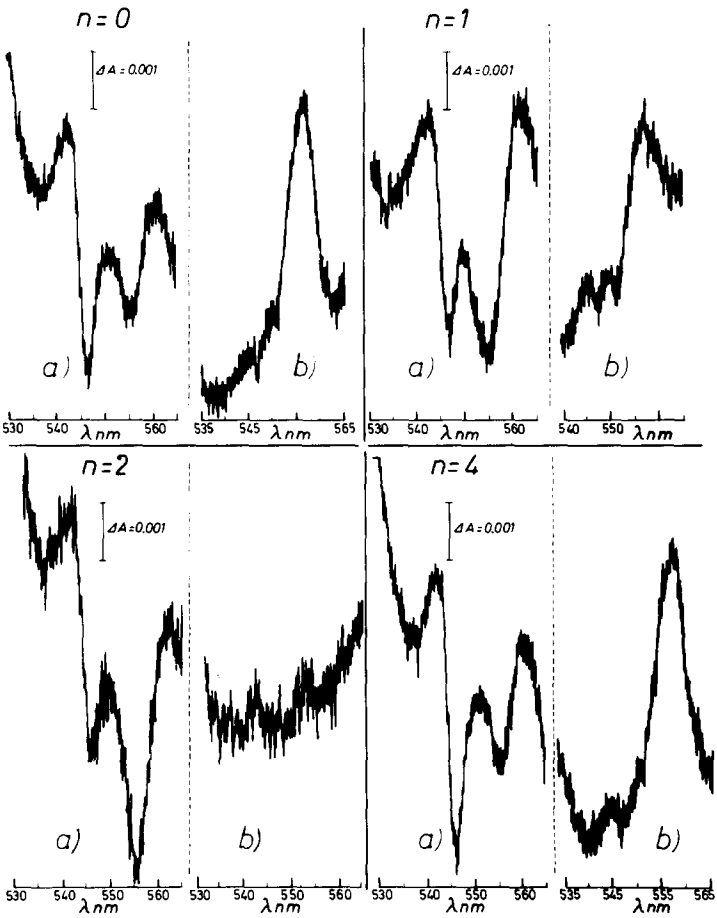


Fig. 1. (a) Difference spectra recorded after illumination of the sample cuvette by 10 s of white light, at −55 °C, after n preilluminating flashes given at room temperature. (b) Same as (a), but after a saturating illumination by continuous white light of the reference cuvette, at −196 °C. Chlorophyll concentration: about 390 $\mu g \cdot ml^{-1}$ for all the spectra.

For the series of experiments partly reported in Fig. 1, we measured the amount of oxidized cytochrome b_{559}. That amount, corrected for small variations of the chloroplast concentration, is plotted *versus* n in Fig. 2 as a percentage of the maximum value obtained for $n=2$. The percentage of oxidized cytochrome b_{559} oscillates with a period of four according to the number n of preilluminating flashes. Due to some damping, no oscillation is observed around $n=18$. The series reported in Fig. 2 is fairly typical: for $n=0$ the percentage varies between 25 and 40 for individual values in 12 experiments. The partial oxidation of cytochrome b_{559} by illumination of dark-adapted chloroplasts ($n=0$) is probably not due to insufficient dark adaptation. Indeed, it is not affected by varying the duration of the dark adaptation (4 or 15 min), and much care was taken to ensure complete darkness during that time. A pattern similar to that of Fig. 2 was observed with chloroplasts obtained from A. and P.

Joliot, kept frozen in solid CO_2, and checked to display the usual oscillatory behaviour in oxygen evolution excited by short saturating flashes.

Illumination by flashes at −55 °C

Excitation of chloroplasts by at least 10 saturating flashes at −55 °C always produces the same final result as continuous light, in terms of reduced C-550 and oxidized cytochrome b_{559} for any value of n. As we have previously shown[15], this is not the case at −196 °C. Additional information can, however, be obtained from the effect of the first flash.

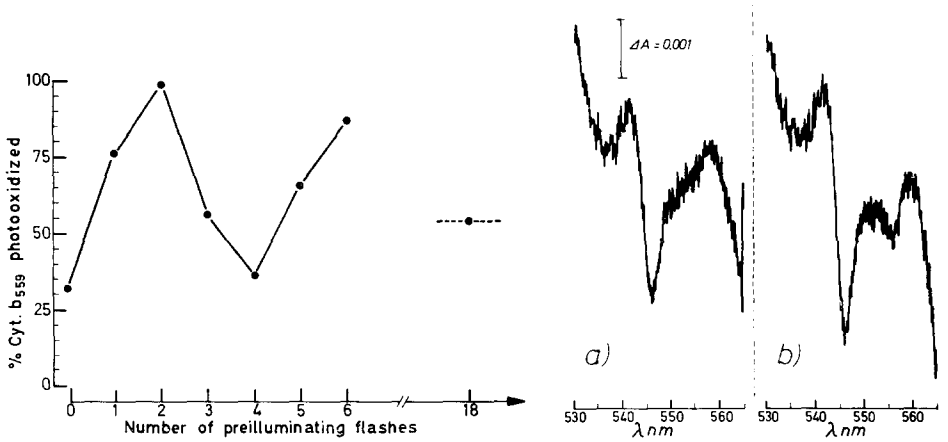


Fig. 2. Percentage of cytochrome b_{559} oxidized by illumination (as Fig. 1a) *versus* the number n of preilluminating flashes. All the data are from the series of experiments partly reported in Fig. 1.

Fig. 3. (a) Difference spectrum recorded after excitation of the sample cuvette by one saturating flash, at −55 °C. No preillumination ($n=0$). (b) Same as (a), after a saturating illumination of the sample cuvette at −196 °C. Chlorophyll concentration: 420 $\mu g \cdot ml^{-1}$.

For dark-adapted chloroplasts, one saturating flash at −55 °C reduces 75% of the C-550 (individual values lie between 70 and 80%). The oxidation of cytochrome b_{559} is very low (less than 10%) and cannot be measured (Fig. 3a). An identical result is obtained after a four-flash preillumination.

After a two-flash preillumination (at 21 °C), one saturating flash (at −55 °C) can reduce only 30% of the C-550. Cytochrome b_{559} is partly oxidized in the same reaction (Fig. 4a). An average value for eight experiments is 18 (±5)%; it is significantly lower than the percentage reduction of C-550. Similar results are obtained for $n=6$. After a preillumination with $n=1$, 3 or 5 flashes the results are intermediate between those with $n=0$ and $n=2$.

Once a saturating flash has been given at −55 °C, and for any value of n, subsequent illumination produces in parallel reduced C-550 and oxidized cytochrome b_{559}. This is true for flash excitation at −55 °C, for illumination in continuous light at −55 °C and for illumination in continuous light at −196 °C (Figs 3b and 4b).

Experiments in the presence of ferricyanide

The experiments previously described have been repeated in the presence of 0.4 mM of potassium ferricyanide which, added to both the sample and the reference

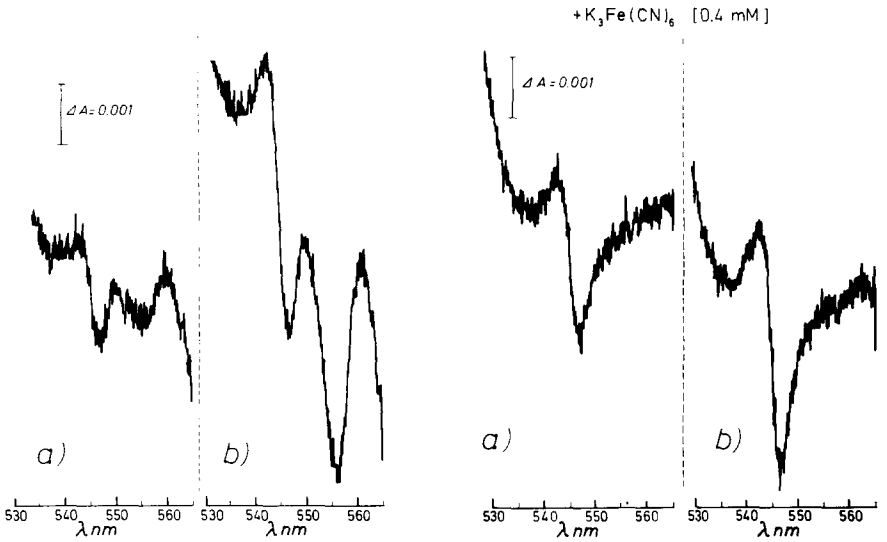


Fig. 4. (a) Difference spectrum recorded after excitation of the sample cuvette by one saturating flash, at −55 °C, following a two-flash preillumination ($n=2$). (b) Same as (a), after a saturating illumination of the sample cuvette at −196 °C. Chlorophyll concentration: 420 $\mu g \cdot ml^{-1}$.

Fig. 5. (a) Difference spectrum recorded after illumination of the sample cuvette by 10 s of white light, at −55 °C. Dark-adapted chloroplasts ($n=0$) in the presence of 0.4 mM of ferricyanide. (b) Same as (a), after a saturating illumination of the sample cuvette at −196 °C. Chlorophyll concentration: 380 $\mu g \cdot ml^{-1}$.

cuvettes, completely oxidizes cytochrome b_{559} and cytochrome *f*. In light *minus* dark difference spectra, no further oxidation of these cytochromes was detected (Fig. 5). The addition of ferricyanide had little influence on the photoreduction of C-550. The same oscillatory pattern, with a period of four *versus n*, is observed for the effect of the first flash at −55 °C. The single difference is that only 65% of the amount of C-550 reduced in the same conditions without ferricyanide can be trapped in the reduced form, after illumination in continuous or in flash light at −55 °C (Fig. 5a). A complete reduction of C-550 is observed following further illumination at −196 °C (Fig. 5b).

DISCUSSION

The experiments reported here point out a correlation between the photoreactions occurring at −55 °C (their yield in flash excitation; the nature of the electron donor) and the state of the reaction centre of Photosystem II, a state which is determined by the conditions of preillumination and which is stabilized by rapid cooling[16]. A comparison of the effect of one flash and of illumination by continuous light at −55 °C leads us to consider that two types of photochemical behaviour can occur for Photosystem II. We suggest that they respectively correspond to two configurations (C_h and C_l) of the reaction centre. Configuration C_h is characterized by a high yield for the reduction of C-550 by flashes, practically 100% by one flash

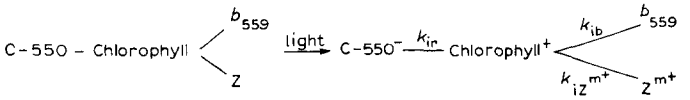
at −55 °C. In that case, the electron donor is not detected by our technique and will be called Z, in agreement with a previous notation[1]. Configuration C_l is characterized by a low photoreduction of C-550 by one saturating short flash at −55 °C: about 20% by the first flash. In that case, the electron donor is cytochrome b_{559} of high potential. The mid-potential form of cytochrome b_{559} (refs 18, 19) is oxidized in dark-adapted chloroplasts. A comparison with the situation occurring in mitochondria (see refs 20 and 21) led us to suspect that the effect of preillumination might influence the distribution of cytochrome b_{559} between the mid-potential and the high-potential forms. However, the redox state of cytochrome b_{559} is not affected by the preillumination, as evidenced by our control experiments without illumination at −55 °C. Another argument for the nonparticipation of the mid-potential form of cytochrome b_{559} is that our results are unchanged by the addition of 40 mM of sodium ascorbate, which reduces this cytochrome (data not shown).

An inspection of our results (Figs 1a, 2 and 3) leads to the conclusion that, in dark-adapted chloroplasts, about 70% of reaction centres 2 are in the configuration C_h and 30% in the configuration C_l. After a two-flash preillumination, we find about 10% of the reaction centres 2 in the configuration C_h and 90% in the configuration C_l. Fig. 2 can be read as indicating that a factor controlling the relative proportion of C_h and C_l configurations is the number of oxidizing equivalents accumulated on the oxygen side of reaction centre 2. Accepting the model proposed by Kok *et al.*[2], we would say that the states S_0 and S_1 (in maximum concentration for dark-adapted chloroplasts or after a four-flash preillumination) favour configuration C_h, and that the states S_2 and S_3 (in maximum concentration after a two-flash preillumination) favour configuration C_l. A similar pairwise grouping of the states has been showed by several authors to be operative for the description of several light emission properties of chloroplasts[5,22,23]. However, it should be pointed out that with the dark-adapted chloroplasts, totally in the states S_0 and S_1 (ref. 22), only 70% of the reaction centres 2 are in the configuration C_h. This low percentage might be due to 30% of the reaction centres being inactive for oxygen evolution at room temperature, or to an effect of low temperature on the relative proportion of the two configurations. In our previous experiments with chloroplasts in the presence of 66% of glycerol, we observed an effect of preillumination, increasing the photooxidation of cytochrome b_{559} at −50 °C, but we did not observe any reversion after a four-flash preillumination. Such a result may indeed be expected, as high concentrations of glycerol inhibit oxygen evolution (Bouges-Bocquet, B. and Joliot, P., personal communication).

Experiments by Erixon and Butler[14] showed a good parallelism between the variable fluorescence and the compound C-550 in chloroplasts. This correspondence can be extended by comparing our results to those of Joliot and Joliot[5]. It appears that fluorescence induction can be effected by one flash (at −50 °C), in conditions where we find that C-550 can be reduced by one flash (C_h configuration). A fluorescence induction partly effected by one flash and displaying a poor yield in continuous light corresponds to a situation where C-550 is only partly reduced by one saturating flash (C_l configuration), and where cytochrome b_{559} is the electron donor. The comparison cannot be extended too precisely, because the identification of oxidized C-550 with the fluorescence quencher is still questionable[24–27].

In order to account for both the switch in the nature of the electron donor

and the variable efficiency of the light reaction (especially in flash excitation), one might consider that the rate constants of the reactions occurring at reaction centre 2 have different values in the two configurations C_h and C_l, as indicated by the index i, with respective values h or l, in the following scheme:



where k_{ir} is the rate constant for a back reaction, k_{ib} and $k_{iZ}{}^{m+}$ for electron transfer from cytochrome b_{559} and Z^{m+}, respectively. With such a model, our results can be explained by supposing that, at -55 °C:

$$k_{hZ^{m+}} \gg k_{hr} + k_{hb} \text{ and } k_{lr} > k_{lb} \gg k_{lZ^{m+}}$$

In spite of the qualitative explanatory value of the scheme, its quantitative use is precluded because none of the rate constants have a known value. An evaluation of k_b made by Floyd *et al.*[7] must be considered with care, since it was obtained in conditions where specific flash effects could occur.

The experiments performed in the presence of ferricyanide indicate that an oscillatory pattern, with a period of four, can occur when cytochrome b_{559} is fully oxidized. This observation is in agreement with a report by Cox and Bendall[28], who conclude that cytochrome b_{559} is not directly involved in oxygen evolution. Our experiments indicate that cytochrome b_{559} is an intrinsic part of reaction centre 2, but they throw little light on the biological role of this molecule.

As regards to the normal photosynthetic reactions occurring at room temperature, the significance of our results remains to be established. One saturating short flash is able to effect a complete photoreaction at room temperature, in either state of Photosystem II[1,2]. The same is not true for the reduction of C-550 at -55 °C in the C_l configuration. This could be due to an effect of temperature on several kinetic parameters, or to the fact that different primary reactions are involved. We present a minimum model to account for our results. A more developed model will require additional information about the donor Z; it will also be necessary to decide if C-550 is the unique electron acceptor or if, as it has been proposed[16], several electron acceptors are normally operating at the Photosystem II reaction centre.

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