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STUDIES ON THE WATER-OXIDIZING SYSTEM BY THE EFFECTS OF DIFFERENT TREATMENTS IN CHLOROPLASTS

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Treatments such as trypsinization (50 μg/ml per mg Chl for 1 h), osmotic shock of the chloroplasts or mild heating altered the oxygen evolution in such a way that the properties of the Photosystem II were simplified. After these treatments, the damping of the oscillation pattern of O_2 yields induced by a flash series remained the same, irrespective of the level of inhibition induced by the treatment. This damping did not decrease with increasing flash energy, as observed in untreated chloroplasts. The light saturation curve of the $S_2 \rightarrow S_3$ transition of the O_2 evolving system no more exhibited the slow-increasing phase at high flash energy observed under normal conditions. The kinetic properties of the O_2-evolving system were also simplified. After the treatments cited above, deactivation of S_2 and S_3 were identical and accelerated with respect to untreated chloroplasts. Turnover kinetics of the transitions $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$ were also similar and simpler without a lag for $S_2 \rightarrow S_3$. These results indicate that the treatments mentioned above disconnect one donor from the O_2-evolving complex. This donor, under normal conditions, contributes to the increase of the quantum yield of the transition $S_2 \rightarrow S_3$ at high flash energy. This donor is here denoted by D. Our results are in agreement with the following working hypothesis: the large miss, observed on the $S_2 \rightarrow S_3$ transition without any contribution of the donor D, may be due to the fact that the system needs a conformation change of the O_2-evolving complex in the S_2 state, so that the main donor Y can oxidize the second H_2O molecule in the water-splitting complex. In the inactive state corresponding to the absence of a conformation change, the donor D, being different in configuration, is likely to oxidize the S_2 state into an S_3 state at high light intensity.

Introduction

Photosynthetic oxygen evolution occurs through five intermediate oxidation states. These states are denoted S_i ($i = 0$–4), where i is the number of electrons removed from the water-splitting complex by PS II [1,2]. The states S_0 and S_1 are stable in the dark. The S_2 and S_3 states are stable over 500 ms after a flash and then they deactivate with a lifetime of about 40 s [2].

Several electron donors have been shown to be involved in the pathway leading to oxygen evolution (see the review by Bouges-Bocquet [3]). P-680, a chlorophyll *a* [4], is the primary donor of PS II and is reduced in a reaction of $t_{1/2} = 50$ ns after the first flash of a series on dark-adapted chloroplasts [5]. A slower reduction is observed after subsequent flashes with a rate which is somewhat slower for the transitions $S_2 \rightarrow S_3$ and $S_3 \rightarrow O_2$ than for the other transitions [6]. At liquid nitro-

Abbreviations: Chl, chlorophyll; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; PS II, Photosystem II, Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; cyt, cytochrome.

gen temperature, an oxidation of both cyt *b*-559 and P-680 are reported [7]. Above −65°C electron transfers from S_0 and S_1 to P-680 may occur [8] and the electron transfers from cyt *b*-559 no longer take place when the centers are in state S_0 or S_1 before cooling.

Signal II, an EPR signal probably due to a semiquinone cation [9], can under some circumstances be photoinduced at a low temperature [10]. At room temperature a compound giving rise to Signal II very fast (Signal II_{vf}) is called Z [11,12], and is shown to be the immediate donor to P-680 [13].

The presence of several donors in PS II have often been suggested [14,15]. In this paper, experimental evidence is given of the heterogeneity in the structure of the donors participating in oxygen evolution. A working hypothesis for the structural organization of the O_2-evolving system is elaborated in which either the main donor Y or the auxiliary donor D acts as an intermediate between the water-oxidizing complex and P-680. Therefore, these donors do not correspond to any Y [16,17] or D [18,19] models published so far. However, in some centers, these donors operate as Z_1 and Z_2 of Bouges-Bocquet [3].

Materials and Methods

Fresh chloroplasts (thylakoids) were prepared from market lettuces as in Ref. 20 and suspended in a medium containing 0.4 M sucrose/10 mM NaCl/3 mM $MgCl_2$/50 mM *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (Tricine), buffered at pH 7.8.

The rate electrode used for oxygen flash yield measurements has been previously described [20].

The fluorescence intensity (yield) of Chlorophyll *a* was measured 80 ms after each flash of a sequence (spacing 400 ms) with a very weak green light emitted for 6 ms (emitting diodes, Hewlett Packard 5082/4958). The flashes, the blocking of the photomultiplier (EMI 9558 B) and the weak measuring light were triggered by a microcomputer (Apple II plus).

Chlorophyll *a* fluorescence signals after each flash in a series of 16 flashes were first amplified, then recorded in a transient waveform recorder (Physical Data Incorporation 523-A) and then stored in the microcomputer and finally printed on a printer for the final figures. The fluorescence yields (F_V) were printed out and subtracted from F_0 the fluorescence yield after darkness.

Excitation of photosynthesis was provided by 'Stroboslave' general radio flash lamps (half-rise time, 3 μs).

Results

Trypsin, a large water-soluble enzyme, primarily attacks proteins on the outer side of the thylakoid membrane and more especially a proteinaceous component covering the primary quinone acceptor Q_A, thus making Q_A accessible to the exogenous oxidant ferricyanide [21]. According to Steinback et al. [22] and Oettmeier [23], trypsin also prevents some inhibitors from binding at the secondary electron acceptor site (Q_B). We have already used these properties in a previous work [24] and observed that trypsin, at a concentration of 5 μg/ml per mg Chl in the presence of 0.5 mM ferricyanide, was sufficient to make the chloroplasts resistant to 10 μM DCMU. The damping of the oscillations of the oxygen yield induced by a flash series was not changed in these conditions. When we treated chloroplasts with a higher concentration of trypsin (generally 50 μg/ml per mg Chl for 1 h), we observed two phases as a function of time, independently of the rate of inhibition which varied according to the batches. In the first phase (varying from 10 to 40 min), the effect of trypsin consisted in a progressive increase of the damping of the oscillations in the oxygen flash yield pattern. However, in the second phase, there was no change in the characteristic oscillations, even after a severe trypsin treatment (0.5 mg/ml per mg Chl) which decreases the overall amplitudes of the oxygen yield by 70%. This suggests some heterogeneity in the structure of the oxygen evolving complex.

In untreated chloroplasts, as shown in Fig. 1A, the light-saturation curve of the $S_2 \rightarrow S_3$ transition of the oxygen-evolving system exhibited a second phase where the yield increases slowly with flash energy. This was experimentally measured by the variation of the oxygen evolved on the third flash of the series as a function of the energy of the second flash. There is no change of the slow

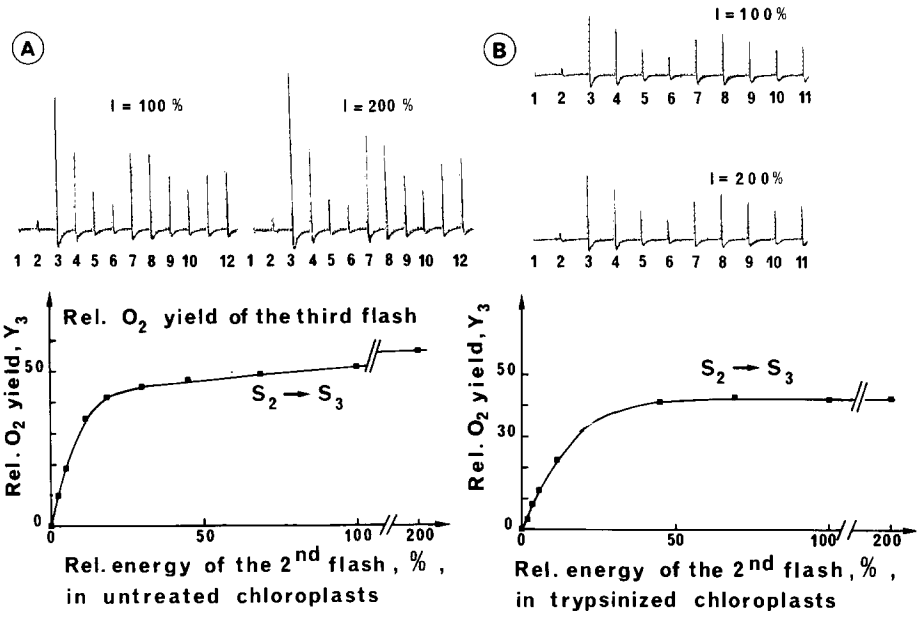


Fig. 1. O_2 flash yield patterns (top) and light saturation curves of the transition $S_2 \rightarrow S_3$ (bottom) in untreated lettuce chloroplasts (A), and in trypsinized chloroplasts (B). The numbers on the abscissa on the top curves indicate the number of flashes. Bottom, O_2 evolved on the third flash (Y_3) (ordinate) of a series of flashes as a function of the energy of the second flash I_2 (abscissa); 200% was the relative light energy for two simultaneous identical flashes. Spacing between flashes, 400 ms. Dark adaptation, 5 min. In untreated chloroplasts (A), the analysis of the O_2 yield series, according to the theory of unequal misses [26], gives the following S_i state distribution ($i = 0$–3) for flashes of a relative energy of 100%: 0, 0, 0.52, 0; for flashes of a relative energy of 200%: 0, 0, 0.465, 0; in trypsinized chloroplasts (B), the miss distribution is 0, 0, 0.54, 0 for both $I = 100\%$ and $I = 200\%$.

increase with the duration of the flash. A short 300 ns, or a larger 3 μs flash does not change the light-saturation curve [25], simply because the turnover time of $S_2 \rightarrow S_3$ is fundamentally larger ($t_{1/2} = 500$ μs). The other transitions $S_1 \rightarrow S_2$ or $S_3 \rightarrow O_2$ are light saturated with the high flash energies [26].

As shown in Fig. 1A, a two-fold increase of the flash energy significantly decreases the damping of the oxygen flash yield pattern. Calculations of the miss and double-hit percentage by the least-square fit indicate a decrease of the miss as the relative flash energy is increased from 100 to 200%. This result is deduced from either the unequal miss model [26] or the equal miss and double-hit model [27]. According to our method [26], it was found that the miss on the S_2 state decreases from $\alpha_2 = 0.52 \pm 0.005$ to $\alpha_2 = 0.46 \pm 0.005$ as the flash is increased two-fold. According to Jursinic's method [27], the miss and double-hit parameters $\alpha = 0.18$ and $\beta = 0.04$ found in the 100% energy pattern in Fig. 1A are decreased to $\alpha = 0.139$ and $\beta = 0.036$ in the 200% energy pattern. The conventional analysis with equal miss does not show a corresponding increase of double hit with flash energy, but on the contrary a small apparent decrease is obtained. Therefore, Fig. 1A shows that the second increasing phase of the light-saturation curve of $S_2 \rightarrow S_3$ is associated with a corresponding decrease of the damping of the oscillation pattern, independently of the details of model used to fit the flash yields.

In chloroplasts treated with trypsin (50 μg/ml per mg Chl), the oscillation pattern of oxygen flash yields was more damped than in untreated chloroplasts and the damping of the oscillation pattern could not be decreased with increasing flash energy (the unique miss $\alpha_2 = 0.535 \pm 0.005$ according to Ref. 26, $\alpha = 0.19$, $\beta = 0.04$ according to Ref. 27; see Fig. 1B). In correlation with this unchanged pattern as a function of flash energy, the light-saturation curve of the transition $S_2 \rightarrow S_3$ became monophasic, independently of the flash energy for energies higher than 3–5 photons per

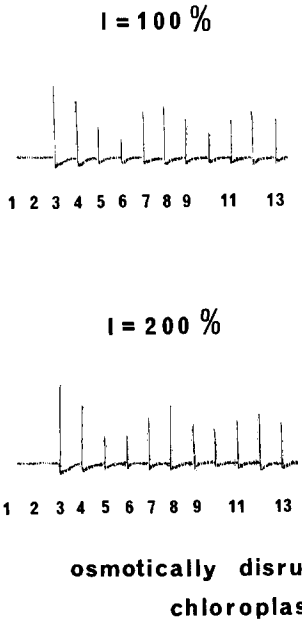


Fig. 2. O_2 flash yield patterns (ordinate) in dark-adapted (5 min) lettuce chloroplasts which were osmotically disrupted in hypotonic buffers (2 mM Hepes/2 mM EDTA, pH 8). The numbers on the abscissa indicate the number of flashes. 200% was the relative energy of two simultaneous flashes; flash spacing, 400 ms. Analysis of the O_2 yield sequence according to a theory of unequal misses [20] gives the following S_i state distribution ($i = 0$–3): 0, 0, 0.535, 0.

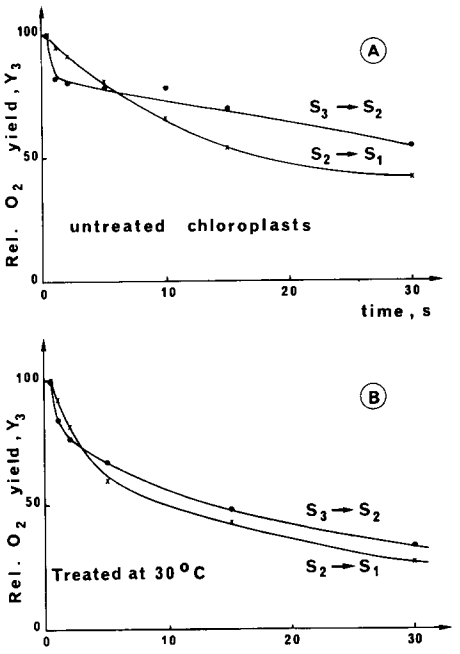


Fig. 3. Deactivation of S_3 and S_2 in untreated lettuce chloroplasts at 20°C and those treated at a constant temperature of 30°C (1 h). For kinetics of S_3, the time was varied between the second and the third flash of a sequence and the O_2 detected after the third flash of the series; for kinetics of S_2, the time was varied between the first and the second flash of a sequence and the O_2 detected after the third flash of the series. Other flashes were spaced 400 ms apart. Dark adaptation, 5 min.

center (evaluated from the saturating-light intensity of the other S_i state transitions). This result confirms that the second phase in the light-saturation curve of the transition $S_2 \rightarrow S_3$ in untreated chloroplasts is necessarily related to a decrease of the damping of the oscillation pattern.

Other treatments are able to induce the same effect as described above. One example is the mild heating of the chloroplasts at a constant temperature of 30°C. Aging induced by an increase of temperature has been extensively studied [28,29]; and recently Khanna et al. [30] have found that at 30°C a part of the very highly bound manganese is released. Another treatment used was an osmotic shock of the chloroplasts in the presence of 2 mM Hepes/2 mM EDTA, pH 8, a procedure similar to that in Ref. 31 (except that no oxidizing agents were added). According to Abramowicz et al. [31], this procedure was efficient in releasing a 34 kDa manganese-binding protein. After these treatments, the damping of the oxygen flash yield pattern increased to a certain extent, but it did not change at any level of inhibition. Furthermore, the damping and the characteristics of the oscillation pattern were similar under the three treatments used. As shown in Fig. 2 the oscillation pattern of oxygen yields in osmotically disrupted chloroplasts did not change as the flash energy was increased two-fold. It was identical to that in trypsinized chloroplasts (Fig. 1B) or that in chloroplasts heated to 30°C (not shown) with the same value of misses.

The kinetic properties of samples treated as described above were studied. The mild treatments used did not modify the stability of the S_2 and the S_3 state centers at short time s(up to 400 ms). In contrast, at longer time, deactivation of S_3 and S_2 were changed. In untreated chloroplasts, deactivation of S_3 shows two distinct phases in Fig. 3A: a rapid one (2 s) which decreases S_3 up to 15%, and

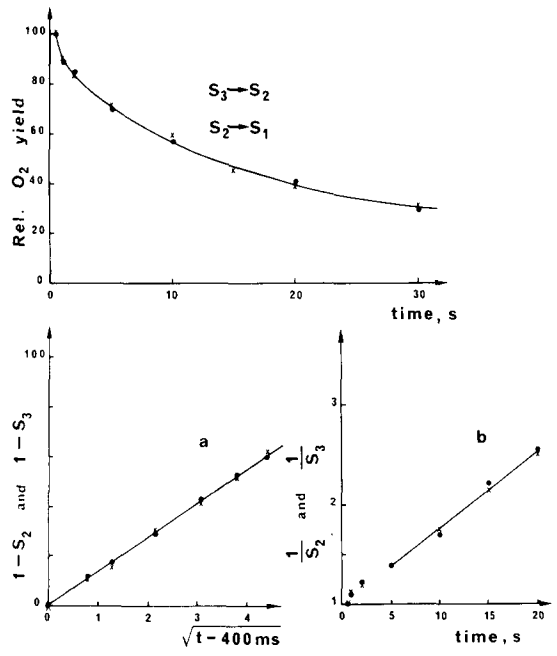


Fig. 4. Deactivation of S_3 and S_2 in lettuce chloroplasts treated with 50 μg/ml trypsin for 1 h and 0.5 mM ferricyanide. Same procedure as in Fig. 3. In (a), the decrease of S_3 and S_2 is plotted as a function of $\sqrt{t-400\text{ ms}}$ during which S_3 and S_2 effectively deactivated. In (b), $1/S_3$ and $1/S_2$ is plotted as a function of the dark time after the second (S_3) or the first flash (S_2).

a slow one. The deactivation curve of S_2 has a smoother shape. The treatments accelerated the deactivation curves of S_3 and S_2, so that the curves became more similar. At a constant temperature of 30°C (Fig. 3B), or in the presence of trypsin (50 μg/ml per mgChl) and ferricyanide (Fig. 4), deactivation curves for S_3 and S_2 were observed to be nearly or completely identical. The simpler deactivation curve observed after trypsin treatment in Fig. 4 shows two different behaviours as a function of time. At longer times, the S_3 and S_2 state centers deactivate in a bimolecular fashion with a second-order rate constant (Fig. 4b). The deactivation of the donor complex involves electron transfer by back reaction from the reduced secondary quinone Q_B [32], which explains the second order kinetics of deactivation [33]. At shorter times (up to 10 s) the shape of the deactivation curve in the presence of trypsin indicates a diffusive process. The amount of deactivated centers is a linear function of the

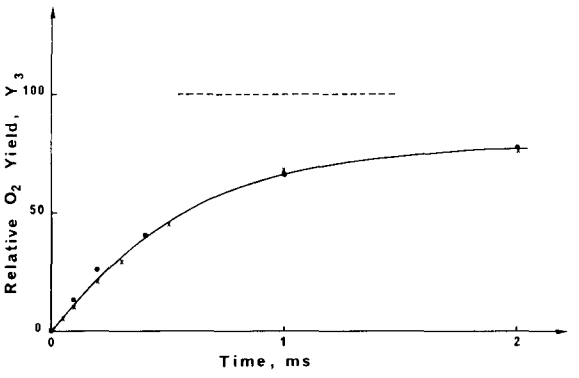


Fig. 5. Turnover times of the transitions $S_1 \rightarrow S_2$ (●) and $S_2 \rightarrow S_3$ (×) in chloroplasts treated with 50 μg/ml trypsin for 1 h and 0.5 mM ferricyanide. Same procedure as in Fig. 3.

square root of the time in Fig. 4a, as if a chemical component (cyt *b* ?, or a carotenoid ?), diffusing from a particular locus of the membrane [34], were deactivating the S_3 and S_2 states.

The turnover kinetics of the S_2 and S_3 state centers with a half-time of 500 μs, were not drastically changed after trypsin treatment (50 μg/ml per mg Chl). However, they appeared more similar to each other: the lag in the turnover kinetics of the transition $S_2 \rightarrow S_3$ observed in untreated chloroplasts [35] was absent after trypsin treatment as shown in Fig. 5.

The effect of a high concentration of trypsin (in the presence of ferricyanide) on the oscillations of period four [36] of the chlorophyll *a* fluorescence yield measured after each flash in a sequence of high-energy flashes ($I = 100\%$) is shown in Fig. 6. In untreated chloroplasts, the maximum fluorescence yield (at 80 ms after the flash) is observed after the first flash and the first minimum after the fourth flash (taking into account the fluorescence increase as a function of flash number due to the progressive inhibition in PS II turnover caused by the filling of the plastoquinone pool). Therefore, the fluorescence oscillations are mainly attributable to the S_2 state. Trypsin induces essentially two effects: (a) it strongly depresses the amplitude of the chlorophyll *a* fluorescence oscillations and (2) in the remaining oscillations it shifts the maximum fluorescence yield from the first to the second flash whereas, as shown previously, the damping of the oxygen flash yield is not tremendously altered in the same conditions.

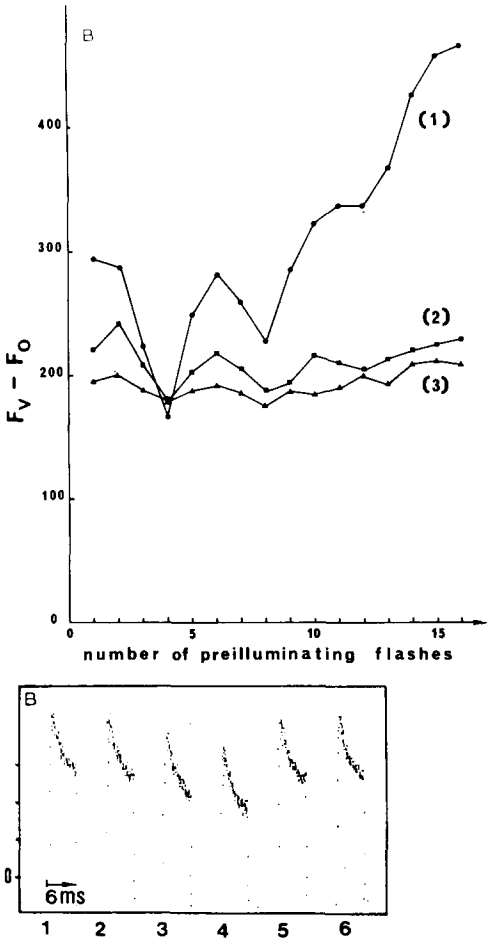


Fig. 6. (A) Fluorescence yields induced by high energy flashes (100%) in dark-adapted chloroplasts, and in the presence of 50 μg/ml trypsin plus 0.5 mM ferricyanide. (1) Dark-adapted chloroplasts and (2) 50 μg/ml trypsin plus 0.5 mM ferricyanide were added to chloroplasts for 10 min; (3) the same treatment as in (2), but for 30 min; $F_V - F_0$: difference between the fluorescence yield after each flash and the fluorescence yield after darkness. (B). Recording of F_V as a function of flash number in the same conditions as in (1).

Discussion

The effects of the treatments used in this work lead essentially to a simplification of the oxygen-evolution properties. A striking property in untreated chloroplasts is the biphasic light-saturation curve of the transition $S_2 \rightarrow S_3$, which is far from being completely saturated when the highest-energy flashes are applied. These treatments (high concentration of trypsin, mild heating, etc.) suppress the second increasing phase of the saturation curve of $S_2 \rightarrow S_3$ which thus becomes saturated at low intensities. This complete saturation is, however, not correlated with a smaller damping.

The observed biphasic saturation curve of the O_2 yield after the third flash as a function of the energy of the second flash cannot be explained by a double advancement in S_i states as $S_1 \rightarrow S_3$. This is firstly because the conventional analysis with equal misses and double hits [27], does not show a corresponding increase of double hits with the increase of the flash energy, as predicted by any model. Thus, there is no correlation between the amount of double hits obtained with the analysis and the presence of the slow increase at high flash energy. Secondly, at the highest flash energy the explanation of the slow increase in Fig. 1A representing double hits implies a very high proportion of double hits (around 0.15–0.20 on $S_1 \rightarrow S_3$) which should have changed the periodicity of the oscillation pattern of oxygen yield [25]. However, this change is not observed experimentally: for example, in Fig. 1A, the periodicity remains clearly 4 even up to the 12th flash.

The origin of the damping of the oxygen yield pattern is a controversial subject. Generally, any reaction participating in oxygen evolution can, if incomplete, induce misses. In the past, we have suggested that a fast equilibrium between an inactive and active state may explain misses [37]. One simple case, as investigated by Vermaas [38], is the equilibrium $Q_A^- \cdot Q_B \rightleftharpoons Q_A \cdot Q_B^-$ which may increase the misses if there is a sufficiently inactive $S_i Q_A^-$ state exchanging with the active $S_i Q_A$ state. This occurs in particular with fast inhibition, for example, due to the fast action of an inhibitor on Q_A. In this case, an equal miss on each state could exist. However, this assumption is open to some criticism.

(1) The existence of a real equilibrium $Q_A^- \cdot Q_B \rightleftharpoons Q_A \cdot Q_B^-$ has not been completely proved and the apparent stability of Q_B observed spectrophotometrically [39,40] could be the result of kinetic rather than thermodynamic factors. Diner and Delosme [41] using PS II particles which lacked the secondary acceptor Q_B found that the presence or absence of Q_B had no effect on the midpoint potential E_m of Q_A/Q_A^- suggesting some independence of Q_A from Q_B. In addition, by displacement of the equilibrium the misses could be sup-

pressed, but this has never been observed.

(2) If the model of equal misses were a reality, the least-square fitting method applied to the oscillation pattern of oxygen yield would not lead to inconsistencies. This method predicts 17% of double hits with equal misses for experimental oxygen yield patterns obtained with a sequence of weak-energy flashes [26]. This result is explained by a curious mathematical property observed in theoretical oxygen yield sequences generated by a computer. Theoretical sequences containing very unequal misses without any double hits correspond to a model of equal misses with a finite proportion of double hits. This amount of double hits simulates the inequality of misses and also increases drastically with the damping of the sequence [26].

(3) An equal miss and double hit model is inconsistent with the two observed phases in the light-saturation curve of the $S_2 \rightarrow S_3$ transition. As is shown experimentally (Fig. 1A) that the $S_2 \rightarrow S_3$ transition is far from reaching complete saturation at usual flash energy, we assume that some proportion of centers in the S_2 state does not undergo the $S_2 \rightarrow S_3$ transition.

Our results after various treatments suggest that a certain damping of the oxygen flash yield pattern is intrinsic to the oxygen-evolving system, and that in untreated chloroplasts a part of the $S_2 \rightarrow S_3$ transitions are performed via the oxidized form of a donor D different from the main donor to P-680. This reaction decreases the damping and increases the yield of this transition.

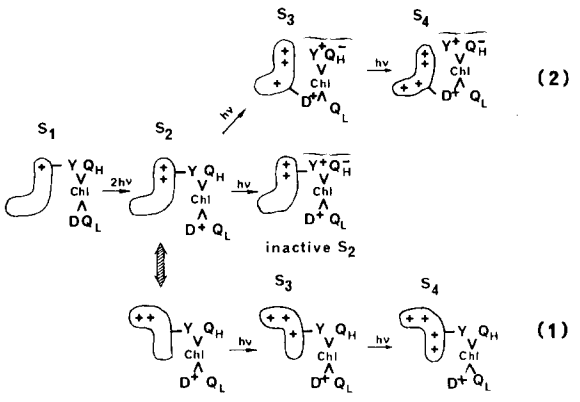
Following the above interpretation for the $S_2 \rightarrow S_3$ transition, we attribute the biphasic deactivation kinetics of the S_3 and S_2 state centers in untreated chloroplasts (Fig. 3A), to the back reaction of oxidizing equivalents stored on the donor side via two parallel donors. The treatments used in this work disconnect one of the donors from the oxygen-evolving complex, leading to monophasic deactivation kinetics. These kinetics are more accelerated in treated than in untreated chloroplasts (Fig. 4). This suggests that the deactivation of the S_3 centers with the auxiliary donor D is much slower than the deactivation of the S_3 centers with only the main donor.

In untreated chloroplasts after several minutes of dark adaptation (3–30 min) a finite concentration of centers in the S_2 state remains, as measured by the oxygen evolved on the second flash in a series. (This yield cannot be interpreted by a double hit because this yield does not increase as the square of the light intensity [20]). The proportion of S_2 state centers which deactivate very slowly in the dark could be related to the amount of transitions $S_2 \rightarrow S_3$ occurring through D in the light before dark adaptation. In complete agreement with this assumption, Figs. 1B and 2 show that little or no oxygen at all is evolved on the second flash in a series after proteolytic or osmotic shock treatment, which is interpreted as suppressing the function of D. In contrast, in the presence of a nearly saturating concentration of DCMU (without any other treatment), the O_2 yield on the second flash of a series is relatively high. Correlatively, the slope of the second phase of the light saturation curve of $S_2 \rightarrow S_3$ is much larger than in untreated chloroplasts [24].

We could ask whether the effect, under discussion, may be related to changes on the acceptor side. For example, the changes observed after trypsinization could be ascribed to a partial digestion of the polypeptide with which the secondary acceptor Q_B is associated. Our previous result in chloroplasts treated with a small concentration of trypsin (5 μg/ml per mg Chl) and ferricyanide (becoming resistant to DCMU) [24] answers this question. No change either in the damping of the oscillation pattern of the oxygen yield, or in the deactivation kinetics was observed. Furthermore, the main effect observed under the treatments used in this work, was related to the light saturation curve of the transition $S_2 \rightarrow S_3$, which cannot be explained by events on the acceptor side at the level of Q_B. The effect on deactivation, identical after three different treatments, must have the same origin and cannot be explained by a single argument which is valid for only one treatment.

A working hypothesis

We propose a model with two distinct acceptor donor systems in the same center [42,43]: the main Y Q_H and the auxiliary D Q_L, in which the two primary acceptors of different midpoint potential Q_H and Q_L [41,44] could be associated with the two donors Y and D [3]. The donor D is associated with Q_L because these donors and acceptors have an auxiliary role (D in our model, and Q_L,



Scheme I. Proposed model explaining the miss in the transition $S_2 \rightarrow S_3$, the possibility for an auxiliary donor (D) to increase the quantum yield of this transition at high flash energy and to take part in O_2 evolution. Chl, a reaction center chlorophyll; Y, D, two alternative immediate donors to Chl^+; Q_H, Q_L, two primary acceptors of different potential. (1) The water splitting protein undergoes a conformation change, a rotation in the S_2 state, so that the main donor Y is then in front of the second H_2O molecule site necessary for O_2 formation. The main donor takes part in transition $S_2 \rightarrow S_3$ with a maximum yield of 0.5. (2) The water-splitting protein remains in the same position in the S_2 state, the main donor Y is inactive; the auxiliary donor being different in configuration may take part with a low probability in the transitions $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_4$.

because a high light intensity drastically increases its concentration [45]). The term 'auxiliary' does not mean that D is on a side pathway. D is an electron donor to P-680, replacing the main donor under certain circumstances (Scheme I).

As observed experimentally, a decrease of the misses is associated with the presence of a second slow increasing phase in the light-saturation curve of the transition $S_2 \rightarrow S_3$. Therefore (independently of the misses on the acceptor side in the hypothesis of their existence) we have revealed in this paper the existence of a miss in the photoreaction $S_2 \rightarrow S_3$ involving the main donor. This miss can be decreased with an auxiliary system. The miss on the main donor seems intrinsic and we suggest that it is due to the fact that the system needs a conformation change (or a relaxation) of the oxygen-evolving site in the S_2 state centers, in order that the main donor Y may be in front of the second H_2O molecule of the O_2-evolving site for the conversion of S_2 into S_3 (see (1) in Scheme I). If no conformation change of the protein occurs, the main donor Y is thus in front of an already occupied site and the S_2 state center is therefore inactive. In the S_2 state, the auxiliary donor D could be situated in front of the water-spitting reaction site, and in this configuration (inactive for Y), it could accept an electron and transfer it to P-680 so that the $S_2 \rightarrow S_3$ transition would occur with a low probability. After the next flash, D would also be involved in the $S_3 \rightarrow S_4$ transition, owing to its privileged position near the reaction site (see 2 in Scheme I). Therefore in this model some centers use only one donor (Y) in the advance from S_0 to S_4, but some other centers use the donor Y in the $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$ transitions and the donor D in the $S_2 \rightarrow S_3$ and $S_3 \rightarrow O_2$ transitions.

The donors Y and D are therefore likely candidates for Signal II species. After the first flash in dark-adapted chloroplasts, two charge stabilization may occur in the center. On the acceptor side two electrons would be transferred to secondary acceptors, Q_B and cyt *b*-563 [46] (probably not in all PS II centers depending on flash energy). On the donor side, the precursor form of donor D may be oxidized into a stable form in the S_2 state, giving rise to a paramagnetic species: the Signal II slow species [47]. (The decay time of Signal II slow has a half-time of the order of 1 h [47].) After the second flash, this 'activated' compound, D, could take part in $S_2 \rightarrow S_3$ transitions.

Our result on deactivation kinetics leads us to suggest that two donors were implicated in the deactivation of S_2 and S_3. The slower component of the multiphasic decay kinetics of Signal II slow [48] may correspond to the very slow S_2 and S_3 deactivation involving the donor D. This interpretation could explain the effect of a flash series on the extent of Signal II slow formation (observed at least 40 s after the flash series) which was found to be proportional to the S_2 and S_3 states according to Babcock and Sauer [47].

According to Babcock et al. [11], Signal II_{vf} is indicative of a component which is rapidly oxidized by PS II and then rereduced while oxidizing the S_i states. This definition corresponds to both donors Y and D. Therefore, we may ask whether there is only one immediate donor to P-680 giving rise to Signal II_{vf} as claimed by Boska and Sauer [13], or

several as in Scheme I. The decay time of Signal II_{vf} is lower than 50–100 μs on the first flash, and around 400 μs and 1 ms on the second and third flash, respectively. It corresponds therefore to the S_3 formation on the second flash and to the S_4 formation on the third flash [12]. These data may be interpreted according to the hypothesis that the two donors are plastoquinone species. The Signal II_{vf} decay time of 50 μs on the first flash could imply that between the oxygen-evolving system and the main donor Y, there is another intermediate compound (not a plastoquinone) which feeds electrons to Y with a half time of 50 μs. The auxiliary donor D, which accepts electrons from the water-splitting complex in the $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_4$ transitions, could be responsible for the Signal II_{vf} on the second and third flash. The model of Boska and Sauer [13] with only one donor to P-680 (Z, a plastoquinone species [9]), does not contradict the experimental results of this paper. However, we suggest that Signal II_{vf} could arise from at least two alternative donors to P-680 for reasons explained below. Renger and Völker [49] have shown that in Tris-washed chloroplasts, the oxidation of Z is coupled with its deprotonization at each flash. Thus, if there were only one plastoquinone species acting as a donor to P-680, one proton linked to the donor Z would be liberated at each flash in untreated chloroplasts. This result is not found according to the experimental proton release pattern observed in a flash sequence in untreated chloroplasts [50,51]. Forster and Junge [52] have observed an unexpected rapid proton release during the $S_2 \rightarrow S_3$ transition, which is characteristic for an additional proton release not produced by water oxidation. Such experimental results cannot be explained in terms of only one donor to P-680. We suggest that P-680 accepts electrons from at least two donors and that one of them could be deprotonized once it is oxidized (for example the donor D of our model in the $S_2 \rightarrow S_3$ transition).

As previously suggested, the treatments used in this work suppress the function of one of the two donors of the oxygen-evolving complex. The detailed reasons for this effect may be different in each treatment. Three polypeptides with molecular masses of approx. 33, 24 and 16 kDa have been revealed to be closely related to the function of the O_2 evolution, and they either play an essential role or have a regulatory function [53,54]. These polypeptides are located on the inner side of the membrane [53,55] and do not retain manganese atoms [55]. It has been proposed that manganese is bound to an intrinsic polypeptide of 34 kDa situated within the thylakoid membrane [55,56,57]. Our results indicate that aging at 30°C first influences the function of donor D, before having other effects which decrease the number of active PS II centers. This suggests manganese may take part in the function of D. Trypsin treatment does not release manganese [58]. It attacks the 32 kDa polypeptide of the acceptor side and could perhaps also digest some amino-acids of the 34 kDa polypeptide situated nearby in the membrane. Trypsin also releases 25 and 27 kDa polypeptides representing apoproteins of the light-harvesting complex [59,60]. This effect could be responsible for the decrease of the fluorescence level [61] and of the amplitude of the fluorescence oscillations. As the fluorescence oscillations did not completely disappear after trypsin treatment, we can assume that the relation between the oxidized chlorophyll and the donor D is not interrupted in the presence of trypsin, the disconnection being rather between the donor D and the water-oxidizing complex.

In the present state of knowledge, it is difficult to make speculative statements about the relationship between the lack of an important photosynthetic function induced by certain treatments and the corresponding structural modifications of the O_2-evolving complex.

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