On the change of the charges in the four photo-induced oxidation steps of the water-splitting enzyme System S

Optical characterization at O_2-evolving complexes isolated from *Synechococcus*

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After dark adaptation of oxygen-evolving PS II complexes, oscillatory absorption changes (stable > 0.5 s) with a periodicity of 4 were detected at 514 nm. They have been correlated with the four oxidation states of the water-splitting enzyme system S. Supposing that the changes are due to electrochromic shifts, they might indicate a positive surplus charge in states S_2 and S_3. This means that the electron release pattern 1:1:1:1 is accompanied by a pattern of charge formation 0:+:+:0 and an intrinsic proton release stoichiometry 1:0:1:2 for the transitions $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, $S_3 \rightarrow S_0$.

Photosynthesis *Photosystem II* *Electrochromic absorption change* *Charge change* *Quaternary oscillation* *S-state* *Proton release pattern*

1. INTRODUCTION

In the primary act of system II of photosynthesis one electron is transferred from the excited Chl-a_{II} (P680) [1,2] to the first stable acceptor, a plastoquinone Q_A (X320) [3–5]. The Chl-a_{II}^{+} extracts in 4 turnovers 4 electrons via at least two electron carriers, D_1 and D_2 [6,7], from the water-splitting enzyme S. Thereby 2 H_2O are decomposed into 4 H^+ and one O_2. In single flashes given to dark-adapted chloroplasts as well as PS II O_2-evolving complexes [8,9] 4 significantly different electron transfer times have been registered in the ns range with regard to the 4 turnovers of Chl-$a_{II}^{+} \longrightarrow$ Chl-a_{II} [6,7]. Under repetitive excitation, Chl-a_{II}^{+} reduction kinetics are multi-phasic [10,11]. These kinetics have been explained quantitatively by a superposition of the 4 different electron transfer times induced with single flashes. The times could be correlated with the oxidation states $S_0 \longrightarrow S_3$ of the enzyme system S. The strong retardation of the electron transfer times to Chl-a_{II}^{+} in states S_2 and S_3 compared to S_0 and S_1 was explained by Coulomb attraction through a positive surplus charge in S_2 as well as in S_3 [6,7]. As a consequence of this result, one should observe a stoichiometric surplus charge variation 0:+:+:0 for the transitions $S_0 \longrightarrow S_1$, $S_1 \longrightarrow S_2$, $S_2 \longrightarrow S_3$, $S_3 \longrightarrow S_0$. In this connection, we looked for electrochromic absorption changes which might indicate this pattern, especially the change of charge in states S_2 and S_3. Oscillatory patterns of different events with periods of 2 and 4 are well known (for review, see [12]). Optical oscillations have been attributed to redox reactions and chemical changes, respectively, of Q, S, and unknown components [13–17].

In order to provide optimal conditions for the observation of surplus charge oscillation, one has to exclude contributions of changes from other events.

Abbreviations: SiMo, silicomolybdate; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; FeCy, ferricyanide; Q, plastoquinone

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2. MATERIALS AND METHODS

1. System I reactions are excluded when isolated O_2-evolving PS II complexes are used [8,9]. 2. Reversible redox reactions of the one-electron carrier, Q_A, Chl-a_{II}, D_1 and D_2 do not exceed a few ms. Their absorption changes are therefore eliminated by measurements performed after $\gg 1$ ms, i.e., ≥ 0.5 s after flash excitation. 3. Electrochromic shifts caused by transmembrane charge separation and transmembrane electric fields, respectively [18], are not indicated in isolated PS II complexes. These particles have no membraneous structure and the charge separation cannot be stabilized, because its recombination takes place in <1 ms through diffusion controlled reorientations of ions in solution [18]. 4. The situation is, however, different for long-lived uncompensated charges as for Q_B with a two-electron capacity. Q_B^- is stable >1 s, until, upon a second photo act, two electrons are accumulated as Q_B^{2-} and released into the PQ pool [13]. Q_B^- is formed in the 1st and odd flashes, the unstable Q_B^{2-} in even flashes. Thus Q_B^- oscillates with a periodicity of 2. The contribution of Q_B is eliminated on the one hand through the fast oxidation with relatively high FeCy concentration (see section 3) and, on the other hand, through addition of DCMU which blocks the electron transfer from Q_A to Q_B, together with the acceptor SiMo which accepts electrons only from Q_A. Thus, under the outlined conditions, only the long-lived S-states (>1 s [19]) should be observable. Those which are uncompensated with regard to surplus charges within the PS II complex might be indicated by electrochromic absorption changes. For this reason, optical changes were measured at 514 nm in a range where usually the dominant electrochromic shifts, especially those of carotenoids, appear due to creation of charges and electric fields, respectively [18,20].

Oxygen-evolving PS II particles from thermophilic cyanobacterium *Synechococcus* sp. were prepared according to [10,11] and stored at -80°C. The particles had a ratio of antenna chlorophyll to Chl-a_{II} reaction centers of 70, as determined by oxygen evolved per flash. The suspension used for measurements contained 4–5 $\times 10^{-8}$ M Chl-a_{II} centers, 0.01 M $MgCl_2$, 0.5 M mannitol, 2×10^{-2} M MES/NaOH (pH 6.8) and 2 mM FeCy or 5.2×10^{-4} M silicomolybdate. The temperature was 20°C.

Optical changes at 514 nm were measured with pulsed measuring light realized through a shutter opened between 2–10 ms. To eliminate the lamp instabilities and obtain a constant level of the baseline over 10–20 s with $\Delta I/I < 10^{-4}$, the construction of a special double beam apparatus was necessary. Therefore, the light source (24 V, 250 W, Osram) was split before the cuvette and while the first beam passed through the cuvette (5.2 cm light path), the second beam was used as reference. Both beams were detected by two selected photodiodes (C30842 from RCA). The intensity of the reference beam was adjusted by means of polarizing filters so as to give practically identical levels. The signal difference created 0.5 s after flash excitation was amplified by a preamplifier constructed by our electronics workshop (gain = 100, 0–20 kHz) and recorded by a digital storage oscilloscope (Nicolet Model 206, equipped with a floppy disc). Excitation was performed by a dye laser (Phase R Model DL-1400) with a flash duration of 0.3 μs at 640 nm (Rhodamin 640). Saturating flash energy was 0.7 mJ/cm^2. Measuring light intensity was 0.2–0.4 mW/cm^2. Experimentally we observed no differences in the oscillatory absorption pattern within the experimental error between 2 and 10 ms pulse duration.

The PS II particles were dark adapted at least one hour before the measurements. Besides oscillatory absorption changes, irreversible changes of the transmission after each flash caused a drift (see e.g., fig.1, bottom). The direction and magnitude of this drift varied from preparation to preparation of the PS II complexes and depended on the kind and concentration of the added chemicals. Optimal concentration of the acceptors was figured out for a nearly constant baseline.

3. RESULTS AND DISCUSSION

In fig.1, top, the registered pattern is shown without flash excitation. After dark times of 0.5 s, the measuring light is switched on for 5 ms. In fig.1, center, flash light is fired at the beginning of each 0.5-s dark period. Fast absorption changes during the 0.5-s dark time are therefore not registered but only long-lived (≥ 0.5 s) levels. A

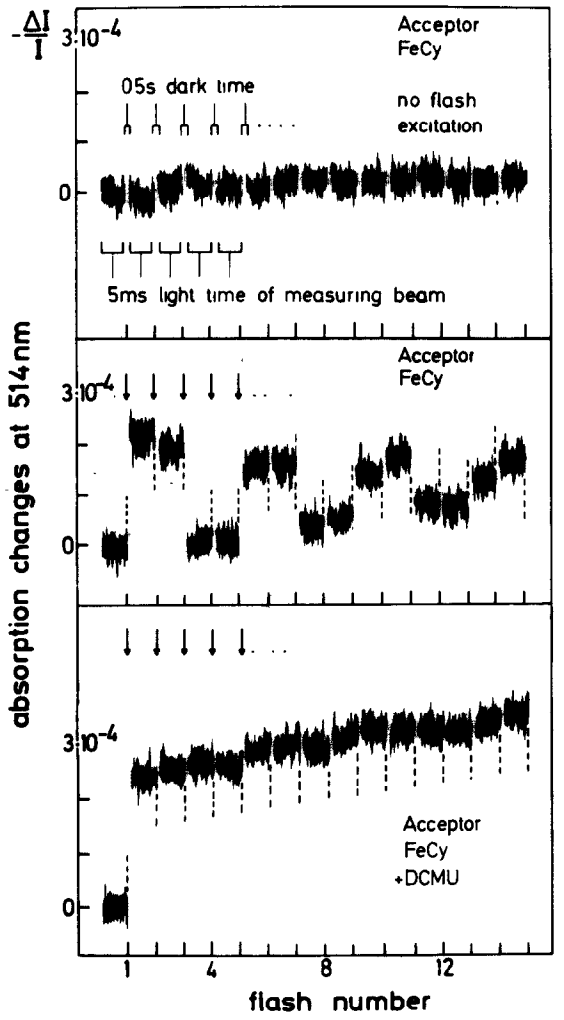

Fig.1. Absorption changes at 514 nm induced by single laser flashes at isolated O_2-evolving PS II complexes as a function of the flash number. Six measurements were averaged. The PS II particles were dark-adapted for at least 1 h prior to measurements. Arrows and dotted lines indicate the position of flash excitation; that is, 0.5 s before the measuring light pulse is switched on and at the end of the 5 ms measuring light pulse, respectively. Top, without flash excitation, FeCy (2 mM); center, with flash excitation, FeCy (2 mM, O_2-evolution in repetitive flash excitation 1 O_2/280 Chl); bottom, with flash excitation, FeCy (2 mM) in the presence of DCMU (2 μM) (no O_2 evolution in repetitive flash excitation).

quarternary oscillation of such absorption change can be seen. After the 1st flash, a relatively large change takes place, which disappears almost completely after the 3rd flash. After the 2nd and 4th flashes nearly no change is observed. With the 5th flash, the quaternary oscillation starts again. Fig.1, center, shows the same experiment performed in the presence of 2 μM DCMU (no O_2 evolution). Besides a jump of absorption after the 1st flash, one can see the disappearance of the oscillations. The drift due to flash-induced irreversible transmission change could not be avoided here. Since DCMU blocks the connection between Q_A and Q_B, the jump after the 1st flash is assumed to be caused by the transfer of one e^- from S to Q_A. The following flashes cannot transfer electrons because Q_A is in the reduced state. Since in the presence of DCMU the initial absorption change after the 1st flash is of the same size as that depicted in fig.1, center, a significant contribution of Q_A^- to the absorption change can be excluded. Addition of dithionite reduces Q_A already in the dark. Therefore, no absorption change is observed even after the 1st flash (not shown).

In fig.2 we used silicomolybdate as acceptor, a substance which accepts electrons directly from Q_A^-. With this acceptor, even in the presence of DCMU (but maximal O_2 evolution), a quaternary oscillatory pattern is observed.

From the practically identical oscillation patterns in fig.1, center, and 2 one has to conclude that $Fe(CN)_6^{3-}$, at the high concentration used (2 mM), is able to oxidize Q_B^- during the dark-time interval of 0.5 s, thus preventing contributions of binary absorption changes due to Q_B. Indeed, by lowering the $Fe(CN)_6^{3-}$ concentration, a more complicated oscillation pattern was observed in the

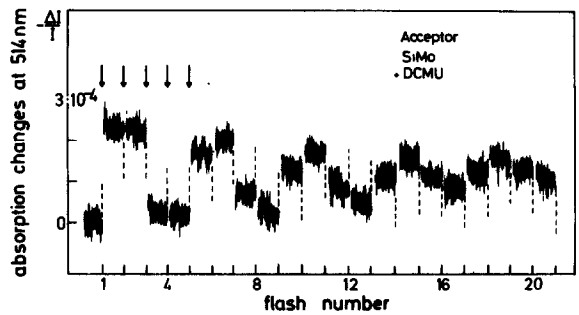

Fig.2. Absorption changes at 514 nm induced by single laser flashes at isolated O_2-evolving PS II complexes with SiMo (5.2×10^{-3} mM) as acceptor in the presence of DCMU (2 μM), as a function of the flash number (O_2 evolution in repetitive flash excitation 1 O_2/280 Chl).

first 4 flashes, probably due to additional oscillation from Q_B/Q_B^- (not shown).

The results can be explained if, with the transition $S_1 \longrightarrow S_2$ of the water-splitting enzyme system S, an increase of the absorption at 514 nm occurs which disappears again with the transition $S_3 \longrightarrow S_0$. The transitions $S_2 \longrightarrow S_3$ and $S_0 \longrightarrow S_1$ do not change the absorption of the PS II complex. According to this assumption the absorption at some particular flash number should be proportional to the sum of S_2 and S_3 populations obtained after that flash. For numerical calculations of the populations of the different S-states we fitted the data on fig.3 according to the equation

$$-(\Delta I/I)_n = k_1(S_2 + S_3)_n + k_2 n$$

considering the 'misses', 'double hits', and the initial S-populations of S_0 and S_1 as variables for calculations of $(S_2 + S_3)_n$ [19]. The term $k_2 n$ considers the drift of the baseline. The best fit of the experimental data was obtained for the values of α = 6.4% for 'misses', β = 1.7% for 'double hits' and S_1 = 100% and $S_0 = S_2 = S_3$ = 0% in the dark-adapted state. In fig.3, top, experimental data (●) are compared with the fitted data (×). In fig.3, bottom, the population of $S_2 + S_3$ states is depicted as a function of the flash number. The agreement in fig.3, top, is good, indicating that with $S_1 \longrightarrow S_2$ an increase of absorption takes place at 514 nm and a decrease with $S_3 \longrightarrow S_0$. It should be mentioned that with decreasing flash excitation energy as well as increasing dark-time interval between flashes (deactivation of the S-states) an increase of the misses is caused so that the oscillations get 'smeared out'. The latter effect permits determination of the deactivation times of the different S-states.

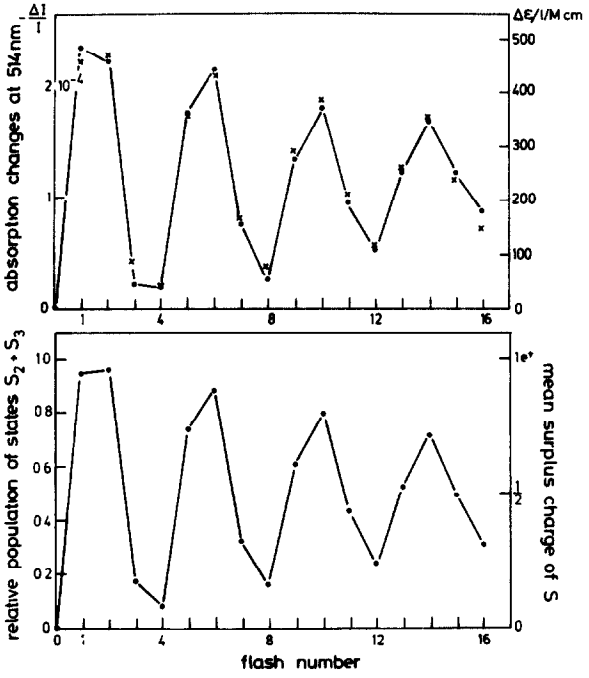

Fig.3. Top, absorption changes at 514 nm (●) after the indicated flash, according to fig.3. The crosses indicate the fit with α-, β- and initial S_0/S_1 values given in the text. $\Delta\epsilon$ is related to the concentration of the reaction centers. Bottom, relative populations of the states S_2 and S_3 after the indicated flash, calculated with α-, β- and initial S_0/S_1 values given in the text. On the right-hand side the mean surplus charge of the enzyme S is depicted, based on the assumption that S_2 and S_3 are positively charged.

Provided that the measured changes at 514 nm are due to electrochromic shifts, i.e., indicating the formation of uncompensated charges within the PS II complex, it follows that with the transition $S_1 \longrightarrow S_2$ formation of a surplus charge occurs which disappears again with the transtion $S_3 \longrightarrow S_0$. The transitions $S_2 \longrightarrow S_3$ and $S_0 \longrightarrow S_1$ do not change the stable charge of the PS II complex. Assuming that the change is realized through a positive elementary charge, e^+, the stoichiometric pattern of charge formation is 0:+:+:0 for the transitions $S_0 \longrightarrow S1$, $S_1 \longrightarrow S_2$, $S_2 \longrightarrow S_3$, $S_3 \longrightarrow S_0$. This pattern can be explained if the electron release pattern 1:1:1:1 is accompanied by an H^+-release pattern 1:0:1:2. For high values of n and in the steady state, i.e., $n \longrightarrow \infty$, $S_0 = S_1 = S_2 = S_3$ = 25%, that is, in the steady state $S_2 + S_3$ = 50%. Under these conditions the surplus charge of the enzyme S is permanently ½ e^+.

The results support the conclusion obtained from the analysis of the ns-reduction kinetics of Chl-a_{II} [6,7]. The consequences in both investigations of an H^+-release pattern 1:0:1:2 further support the results obtained through direct measurements of pH changes in the water phase with single flashes [21–23]. However, it has been claimed that the results on the H^+-stoichiometry obtained by pH measurements in the H_2O phase do not necessarily reflect the intrinsic H^+ release coupled with the 4 oxidation steps. Since our con-

clusions on the H^+-stoichiometry are based on the changes of charges coupled directly with the basic event, our 1:0:1:2 result is not affected by such a restriction and should indicate the intrinsic stoichiometry. It is this stoichiometry which is of importance for examinations of proposed mechanisms of H_2O cleavage.

If the changes at 514 nm are not attributable to electrochromic shifts, one has to consider that these may indicate true absorption changes coupled with chemical changes in states S_2 and S_3; e.g., with the valence change of manganese within the enzyme S: Mn^{3+}/Mn^{4+} ($S_1 \longrightarrow S_2$), Mn^{4+}/Mn^{4+} ($S_2 \longrightarrow S_3$), Mn^{4+}/Mn^{3+} ($S_3 \longrightarrow S_0$). Such an interpretation would match the conclusions on the charge pattern drawn for the case of electrochromic absorption changes. However, with the latter explanation, no conclusions can be drawn about the intrinsic stoichiometry of proton release. Recently, it was discussed that spectral absorption changes in the UV may be caused by an Mn^{3+}/Mn^{4+} transition [24].

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