

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

## Optical characterization at O<sub>2</sub>-evolving complexes isolated from *Synechococcus*

Ö. Saygin and H.T. Witt

*Max-Volmer-Institut für Biophysikalische und Physikalische Chemie, Technische Universität Berlin, Strasse des 17 Juni 135, 1000 Berlin 12, Germany*

Received 2 August 1984

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<sub>2</sub> and S<sub>3</sub>. 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<sub>0</sub>→S<sub>1</sub>, S<sub>1</sub>→S<sub>2</sub>, S<sub>2</sub>→S<sub>3</sub>, S<sub>3</sub>→S<sub>0</sub>.

*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*<sub>II</sub> (P680) [1,2] to the first stable acceptor, a plastoquinone Q<sub>A</sub> (X320) [3–5]. The Chl-*a*<sub>II</sub> extracts in 4 turnovers 4 electrons via at least two electron carriers, D<sub>1</sub> and D<sub>2</sub> [6,7], from the water-splitting enzyme S. Thereby 2 H<sub>2</sub>O are decomposed into 4 H<sup>+</sup> and one O<sub>2</sub>. In single flashes given to dark-adapted chloroplasts as well as PS II O<sub>2</sub>-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*<sub>II</sub><sup>+</sup> → Chl-*a*<sub>II</sub> [6,7]. Under repetitive excitation, Chl-*a*<sub>II</sub><sup>+</sup> 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<sub>0</sub> → S<sub>3</sub> of the enzyme system S. The strong retardation of the electron transfer times to Chl-*a*<sub>II</sub><sup>+</sup> in states S<sub>2</sub> and S<sub>3</sub> compared to S<sub>0</sub> and S<sub>1</sub> was explained by Coulomb attraction through a positive surplus charge in S<sub>2</sub> as well as in S<sub>3</sub> [6,7]. As a consequence of this result, one should observe a stoichiometric surplus charge variation 0:+:+:0 for the transitions S<sub>0</sub> → S<sub>1</sub>, S<sub>1</sub> → S<sub>2</sub>, S<sub>2</sub> → S<sub>3</sub>, S<sub>3</sub> → S<sub>0</sub>. In this connection, we looked for electrochromic absorption changes which might indicate this pattern, especially the change of charge in states S<sub>2</sub> and S<sub>3</sub>. 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, ferri-cyanide; Q, plastoquinone

## 2. MATERIALS AND METHODS

1. System I reactions are excluded when isolated O<sub>2</sub>-evolving PS II complexes are used [8,9]. 2. Reversible redox reactions of the one-electron carrier, Q<sub>A</sub>, Chl-*a*<sub>II</sub>, D<sub>1</sub> and D<sub>2</sub> do not exceed a few ms. Their absorption changes are therefore eliminated by measurements performed after  $\gg 1$  ms, i.e.,  $\geq 0.5$  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 membranous 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<sub>B</sub> with a two-electron capacity. Q<sub>B</sub> is stable  $> 1$  s, until, upon a second photo act, two electrons are accumulated as Q<sub>B</sub><sup>2-</sup> and released into the PQ pool [13]. Q<sub>B</sub> is formed in the 1st and odd flashes, the unstable Q<sub>B</sub><sup>2-</sup> in even flashes. Thus Q<sub>B</sub> oscillates with a periodicity of 2. The contribution of Q<sub>B</sub> 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<sub>A</sub> to Q<sub>B</sub>, together with the acceptor SiMo which accepts electrons only from Q<sub>A</sub>. 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  $-80^{\circ}\text{C}$ . The particles had a ratio of antenna chlorophyll to Chl-*a*<sub>II</sub> 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*<sub>II</sub> centers, 0.01 M MgCl<sub>2</sub>, 0.5 M mannitol,  $2 \times 10^{-2}$  M MES/NaOH (pH 6.8) and

2 mM FeCy or  $5.2 \times 10^{-4}$  M silicomolybdate. The temperature was  $20^{\circ}\text{C}$ .

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  $0.3 \mu\text{s}$  at 640 nm (Rhodamin 640). Saturating flash energy was  $0.7 \text{ mJ}/\text{cm}^2$ . Measuring light intensity was  $0.2-0.4 \text{ mW}/\text{cm}^2$ . 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 ( $\geq 0.5$  s) levels. A

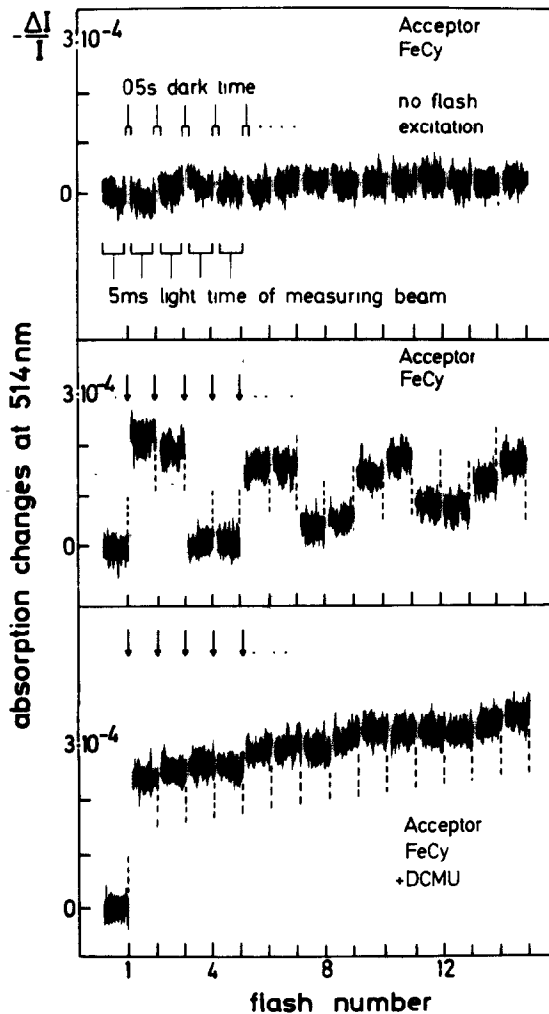


Fig.1. Absorption changes at 514 nm induced by single laser flashes at isolated  $O_2$ -evolving PS II complexes as a function of the flash number. Six measurements were averaged. The PS II particles were dark-adapted for at least 1 h prior to measurements. Arrows and dotted lines indicate the position 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  $\mu$ M) (no  $O_2$  evolution in repetitive flash excitation).

quaternary 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  $\mu$ M 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

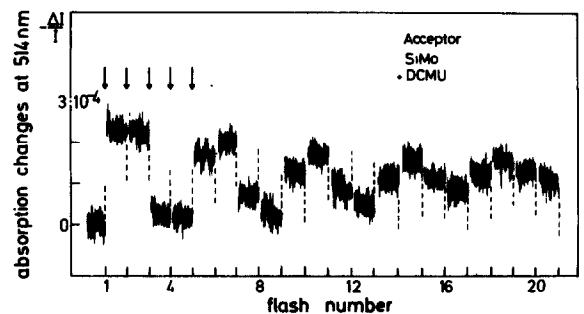


Fig.2. Absorption changes at 514 nm induced by single laser flashes at isolated  $O_2$ -evolving PS II complexes with SiMo ( $5.2 \times 10^{-3}$  mM) as acceptor in the presence of DCMU (2  $\mu$ M), as a function of the flash number ( $O_2$  evolution in repetitive flash excitation 1  $O_2/280$  Chl).

first 4 flashes, probably due to additional oscillation from  $Q_B/Q_{\bar{B}}$  (not shown).

The results can be explained if, with the transition  $S_1 \rightarrow S_2$  of the water-splitting enzyme system S, an increase of the absorption at 514 nm occurs which disappears again with the transition  $S_3 \rightarrow S_0$ . The transitions  $S_2 \rightarrow S_3$  and  $S_0 \rightarrow S_1$  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

$$-(\Delta I/I)_n = k_1(S_2 + S_3)_n + k_2n$$

considering the 'misses', 'double hits', and the in-

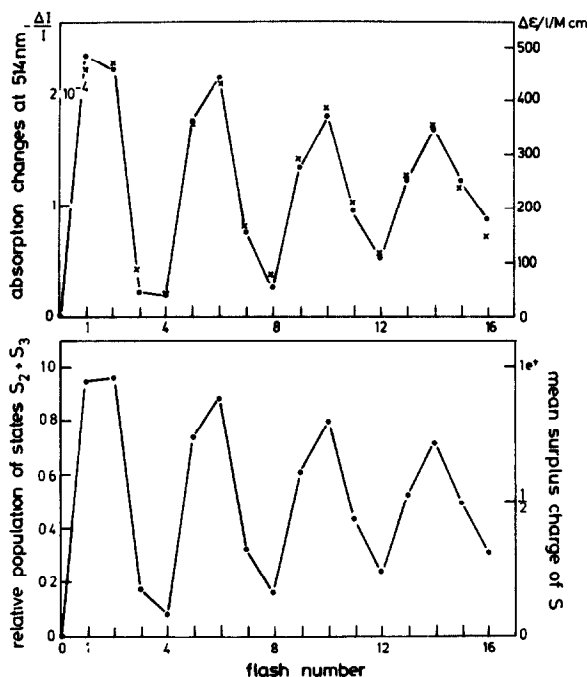


Fig.3. Top, absorption changes at 514 nm (●) after the indicated flash, according to fig.3. The crosses indicate the fit with  $\alpha$ -,  $\beta$ - and initial  $S_0/S_1$  values given in the text.  $\Delta\epsilon$  is related to the concentration of the reaction centers. Bottom, relative populations of the states  $S_2$  and  $S_3$  after the indicated flash, calculated with  $\alpha$ -,  $\beta$ - 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.

itial S-populations of  $S_0$  and  $S_1$  as variables for calculations of  $(S_2 + S_3)_n$  [19]. The term  $k_2n$  considers the drift of the baseline. The best fit of the experimental data was obtained for the values of  $\alpha = 6.4\%$  for 'misses',  $\beta = 1.7\%$  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  $S_1 \rightarrow S_2$  an increase of absorption takes place at 514 nm and a decrease with  $S_3 \rightarrow S_0$ . 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.

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  $S_1 \rightarrow S_2$  formation of a surplus charge occurs which disappears again with the transition  $S_3 \rightarrow S_0$ . The transitions  $S_2 \rightarrow S_3$  and  $S_0 \rightarrow S_1$  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  $S_0 \rightarrow S_1$ ,  $S_1 \rightarrow S_2$ ,  $S_2 \rightarrow S_3$ ,  $S_3 \rightarrow S_0$ . 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.,  $n \rightarrow \infty$ ,  $S_0 = S_1 = S_2 = S_3 = 25\%$ , that is, in the steady state  $S_2 + S_3 = 50\%$ . Under these conditions the surplus charge of the enzyme S is permanently  $\frac{1}{2} e^+$ .

The results support the conclusion obtained from the analysis of the ns-reduction kinetics of Chl- $a_1^+$  [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<sup>+</sup>-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<sub>2</sub>O 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<sub>2</sub> and S<sub>3</sub>; e.g., with the valence change of manganese within the enzyme S: Mn<sup>3+</sup>/Mn<sup>4+</sup> (S<sub>1</sub> → S<sub>2</sub>), Mn<sup>4+</sup>/Mn<sup>4+</sup> (S<sub>2</sub> → S<sub>3</sub>), Mn<sup>4+</sup>/Mn<sup>3+</sup> (S<sub>3</sub> → S<sub>0</sub>). 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<sup>3+</sup>/Mn<sup>4+</sup> transition [24].

#### ACKNOWLEDGEMENTS

We wish to thank Ms Geisenheimer for preparing the PS II particles, Dr Buchwald and Mr Wenzel for constructing the detection unit and Ms DiFiore for drawing the figures. This work was supported by grants from the Deutsche Forschungsgemeinschaft/Sonderforschungsbereich 9, Teilprojekt C9. We also wish to thank Professor Dr Schmidt, Universität Bielefeld, who was kind enough to provide the silicomolybdate.

#### REFERENCES

- [1] Döring, G., Stiehl, H.H. and Witt, H.T. (1967) *Z. Naturforsch.* 22b, 639–644.
- [2] Döring, G., Renger, G., Vater, J. and Witt, H.T. (1969) *Z. Naturforsch.* 24b, 1139–1143.
- [3] Stiehl, H.H. and Witt, H.T. (1968) *Z. Naturforsch.* 23b, 220–224.
- [4] Stiehl, H.H. and Witt, H.T. (1969) *Z. Naturforsch.* 24b, 1588–1598.
- [5] Witt, K. (1973) *FEBS Lett.* 38, 116–118.
- [6] Brettel, K., Schlodder, E. and Witt, H.T. (1984) in: *Advances in Photosynthesis Research* (Sybesma, C. ed.), Proceedings of the VIth Intern. Congr. on Photosynthesis, August 1–16, 1983, Brussels, Belgium, pp.I.3.295–I.3.298, Martinus Nijhoff/Dr W. Junk Publishers, The Hague, The Netherlands.
- [7] Brettel, K., Schlodder, E. and Witt, H.T. (1984) *Biochim. Biophys. Acta*, in press.
- [8] Schatz, G.H. and Witt, H.T. (1984) *Photobiochem. Photobiophys.* 7, 1–14.
- [9] Schatz, G.H. and Witt, H.T. (1984) *Photobiochem. Photobiophys.* 7, 77–89.
- [10] Brettel, K. and Witt, H.T. (1983) *Photobiochem. Photobiophys.* 6, 253–260.
- [11] Schlodder, E., Brettel, K., Schatz, G.H. and Witt, H.T. (1984) *Biochim. Biophys. Acta* 765, 178–185.
- [12] Bouges-Bocquet, B. (1980) *Biochim. Biophys. Acta* 594, 85–103.
- [13] Bouges-Bocquet, B. (1973) *Biochim. Biophys. Acta* 314, 250–256.
- [14] Pulles, M.P.J., Van Gorkom, H.J. and Willemsen, J.G. (1976) *Biochim. Biophys. Acta* 449, 536–540.
- [15] Mathis, P. and Haveman, J. (1977) *Biochim. Biophys. Acta* 461, 167–181.
- [16] Velthuys, B.R. (1981) in: *Proceedings of the 5th Intern. Congress on Photosynthesis* (Akoyunoglou, G. ed.) vol.2, pp.75–85, Balaban International Science Services, Philadelphia, PA.
- [17] Renger, G. and Weiss, W. (1983) *Biochim. Biophys. Acta* 722, 1–11.
- [18] Witt, H.T. (1979) *Biochim. Biophys. Acta* 505, 355–427.
- [19] Kok, B., Forbush, G. and McGloin, M. (1970) *Photochem. Photobiol.* 11, 457–475.
- [20] Junge, W. and Witt, H.T. (1968) *Z. Naturforsch.* 23b, 244–254.
- [21] Fowler, C.F. (1977) *Biochim. Biophys. Acta* 462, 414–421.
- [22] Saphon, S. and Crofts, A.R. (1977) *Z. Naturforsch.* 32c, 617–626.
- [23] Förster, V., Hong, Y.-Q. and Junge, W. (1981) *Biochim. Biophys. Acta* 638, 141–152.
- [24] Dekker, J.P., Van Gorkom, H.J., Brok, M. and Ouwehand, L. (1984) *Biochim. Biophys. Acta* 764, 301–309.