THE REACTIVATION OF EPR SIGNAL II IN CHLOROPLASTS TREATED WITH REDUCED DICHLOROPHENOL-INDOPHENOL : EVIDENCE AGAINST A DARK EQUILIBRIUM BETWEEN TWO OXIDATION STATES OF THE OXYGEN EVOLVING SYSTEM

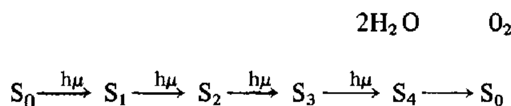
B. R. VELTHUYS and J. W. M. VISSER

Department of Biophysics, Huygens Laboratory of the State University, Wassenaarseweg 78, Leiden, The Netherlands

Received 10 April 1975

1. Introduction

Dark adapted *Chlorella* or isolated chloroplasts subjected to a series of short saturating flashes show a characteristic pattern of oxygen evolution: a very low yield of the first two flashes, a maximum after the third flash and an oscillation with period 4 [1]. Kok et al. [2] have shown that this pattern is due to the fact that the trapping centers of Photosystem 2 operate independently of each other in the oxidation of water. Each center, or associated catalyst (S), cycles through 5 oxidation states, according to the following scheme:



To explain the high oxygen yield of the third flash it was assumed that besides the 'ground state' S_0, state S_1 is also stable in the dark [2,3]. Later, evidence was obtained suggesting that slowly an equilibrium settles between states S_0 and S_1 in the dark. It was shown that after a long dark time about 75% of the centers are in state S_1, independently of the initial S_1/S_0 ratio [4]. Further evidence for the existence of equilibrium reactions between S_0 and S_1 has been reported by Bouges-Bocquet [5]. She showed that the equilibrium could be chemically displaced to both sides. In the presence of ferricyanide virtually all centers appear to attain state S_1. After incubation with DCIP* and ascorbate the equilibrium between S_0 and S_1 appeared to be displaced in favor of S_0.

In this paper we report some new results about the effect of incubation with reduced DCIP on Photosystem 2. We show that the electron paramagnetic resonance Signal II [6] is abolished by reduced DCIP in the dark. A single flash is sufficient for the reactivation of about 80% of the signal. We will argue that our results do not agree with the hypothesis that the effect of reduced DCIP on the yield of oxygen evolution in a flash series is due to a conversion of S_1 to S_0.

* *Abbreviations:* DCIP, 2-6-dichlorophenol-indophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EPR, electron paramagnetic resonance.

2. Materials and methods

Chloroplasts were prepared from market spinach, as described elsewhere [7], suspended in a medium containing 25 mM *N*-tris(hydroxymethyl)-methylglycine (pH 7.8), 0.4 M sucrose, 10 mM KCl and 2 mM $MgCl_2$ and stored in the dark on ice until use. For measurement of O_2 evolution the medium contained 0.1 M KCl. The chlorophyll concentration in samples used for the EPR measurements was 2.5 mg chlorophyll/ml, for O_2 measurements 0.4 mg/ml. Before each experiment the sample was kept in darkness for at least 20 min. All measurements were performed at room temperature.

EPR measurements were recorded using a Varian E–9 Spectrometer, operating near 9.5 GHz at 20 mW. First derivative spectra were obtained by 100 kHz

modulation of the magnetic field. For kinetic measurements the output of the apparatus was fed into a Nuclear Chicago Model 7100 signal averager. Samples in quartz EPR flat cells (0.1 mm pathlength) could be illuminated through the slotted front side of the cavity by a xenon flash lamp (Ft 230, General Electric, C = 10 μF, V = 2400 V). The duration of the flash was 8 μsec at one-third of the peak.

O_2 measurements were performed as described in [8]. For actinic illumination a xenon flash lamp (C = 1 μF, V = 1500 V, flash duration 2 μsec at one-third of the peak), was used, provided with a filter combination consisting of a Schott BG 18/3 colored glass and a Balzers Calflex C heat reflecting filter, and transmitting light between 420 and 570 nm.

3. Results and discussion

Fig.1 shows that the EPR Signal II spectrum, normally present in untreated chloroplasts (spectrum A) [6], is abolished after incubation with 0.1 mM DCIP and 1 mM ascorbate (spectrum B). After a small series of short saturating flashes the original level of

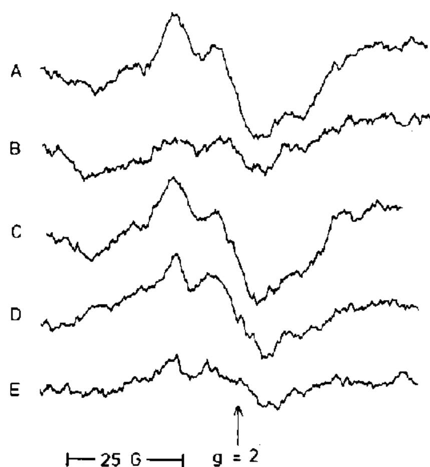


Fig.1. EPR spectra near g = 2 with samples of spinach chloroplasts, kept in the dark for at least one hour. A, control; B, 0.1 mM DCIP and 1 mM ascorbate (incubation time 2 hr); C, same sample as in B after 4 saturating flashes; D, 0.1 mM DCIP and 1 mM $K_3Fe(CN)_6$ (incubation time 3 hr); E, same sample as in B after subsequent incubation with 1 mM $K_3Fe(CN)_6$ for 3 hr. Instrument setting: modulation amplitude 3.2 G; time constant 3.0 sec; scan rate 25 G/min. For further details see text.

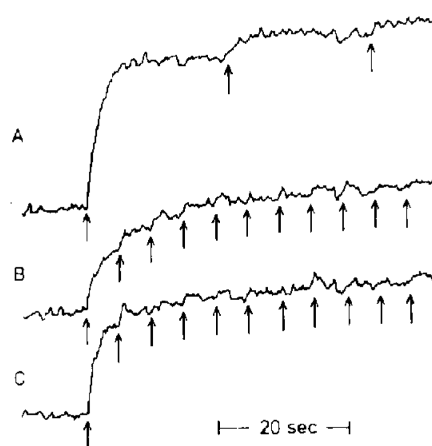


Fig.2. Flash-induced response of Signal II with samples as for fig.1B, except that in (B) 50μM DCMU was added, and in (C) 50 μM DCMU plus 60 mM NH_4Cl. The magnetic field was set at the position of the low field maximum of Signal II (3382 G) with a modulation amplitude of 12.5 G. Instrument time constant: 0.3 sec. Each curve is the average of 7 experiments; a fresh sample was taken for each experiment. Flashes at upward arrows.

Signal II was obtained again (spectrum C). Incubation with DCIP and ferricyanide does not affect Signal II (spectrum D), which shows that the deactivation of Signal II is dependent on reduced DCIP. It may be added that this deactivation seems to be irreversible in the dark: incubation with ferricyanide after the treatment with reduced DCIP did not restore the signal (spectrum E).

In fig.2 the kinetics are shown of the flash-induced restoration of Signal II with chloroplasts incubated with reduced DCIP. The halftime of the increase in Signal II after a flash is about 2 sec. This halftime is similar to that obtained [9] for the restoration of Signal II in untreated chloroplasts prepared from dark-stored spinach leaves, a procedure which gives chloroplasts with a low initial level of Signal II [9,10]. It can also be seen from fig.2A that a single flash restores about 80% of Signal II. As is to be expected from this observation, the main part of Signal II can also be reactivated in the presence of 50 μM DCMU (fig.2B). Under these conditions a single flash is relatively inefficient but in a series of flashes most of Signal II (70%) was reactivated. This inefficiency of a single flash in the presence of DCMU was also observed by Babcock and Sauer [9]. It was explained by them

as being due to a competition between the Signal II precursor and Q^-, the reduced primary acceptor of Photosystem 2, for the oxidation equivalent generated in system 2. This explanation is strongly supported by an experiment in which, in addition to DCMU, 60 mM NH_4Cl was added. As was found recently (B. R. Velthuys, to be published elsewhere), the back reaction between the photo-generated oxidation equivalent and Q^-, the reaction $S_2Q^- \rightarrow S_1Q$, is slowed down by ammonia. It might therefore be expected that the efficiency of generation of Signal II by flashes in the presence of DCMU is enhanced by NH_4Cl. As is shown in Fig.2C, this was indeed observed. The kinetics of formation of Signal II in the absence of DCMU were not affected by ammonia (not shown).

The fact that a one-step S-state transition is sufficient for reactivation of Signal II in most reaction centers argues against the assumption, made on basis of measurements of oxygen evolution [5], that after incubation with reduced DCIP the majority of the centers is in state S_0. A single flash given to centers in state S_0 would transfer these centers to state S_1. These centers then would not be able to produce Signal II, since only centers in states S_2 and S_3 can reactivate this signal [9]. A better explanation for the effect of reduced DCIP on oxygen evolution might be: the change in the oxygen flash-yield pattern is due to the 'loss' of a positive charge, used for the regeneration of Signal II (see also [9]). In order to obtain more evidence for this explanation we have measured the flash-yield of oxygen (Y_n) after incubation with reduced DCIP [5] with different dark times between flashes. Fig.3 shows that the flash-yield pattern is dependent on the flash-interval time: with flashes separated by 0.3 sec the maximum is obtained after the third flash, with a flash interval of 1.0 sec the maximum is obtained after the fourth flash. The second maximum is at the eighth flash, with both flash-frequencies. This behaviour of DCIP-treated chloroplasts is not explained by the assumption [5] that the initial S_1 concentration was decreased after incubation with reduced DCIP. Instead, the results support our suggestion that the low Y_3/Y_4 ratio obtained under these conditions is due to the regeneration of Signal II.

It may be concluded that there is no evidence to support the assumption of Bouges-Bocquet [5] and other investigators [11] that the ratio S_1/S_0 depends on the redox potential of the solution. In our opinion,

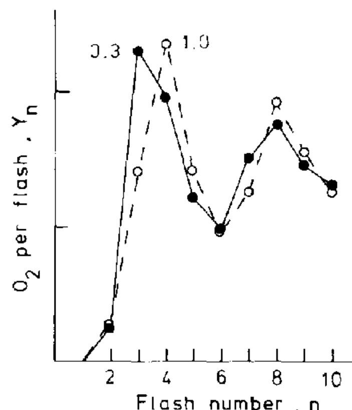


Fig.3. Oxygen flash-yield sequence for spinach chloroplasts after incubation with 0.1 mM DCIP and 1 mM ascorbate. After 15 min incubation the medium in the compartment adjacent to the chloroplast suspension was replaced (because DCIP reacted with the electrode) with medium without DCIP and ascorbate; 6 min later the series of flashes were given. The time between two flashes was 0.3 sec (•) or 1.0 sec (○).

this leaves very little evidence for the assumption that centers may be in state S_0 as well as in state S_1 after dark adaptation. Instead, it may be proposed that all centers revert to state S_1 in the dark. The apparent presence of a small amount of S_0 after a long dark time (in the absence of DCIP) should be reinterpreted then in terms of a relatively high 'miss parameter' (i.e. the fraction of centers of which the S-state is not, or only transiently, converted) in the first flash(es).

Acknowledgements

This investigation was supported by the Netherlands Foundation for Chemical Research (SON), financed by the Netherlands Organization for the Advancement of Pure Research (ZWO). We wish to thank Ir. G. den Haan for his help with the oxygen measurements, and Dr J. Amesz for a critical reading of the manuscript.

References

[1] Joliot, P., Barbieri, G. and Chabaud, R. (1969) Photochem. Photobiol. 10, 309–329.
[2] Kok, B., Forbush, B. and McGloin, M. P. (1970) Photochem. Photobiol. 11, 457–475.

[3] Forbush, B., Kok, B. and McGloin, M. P. (1971) Photochem. Photobiol. 14, 307–321.
[4] Joliot, P., Joliot, A., Bouges, B. and Barbieri, G. (1971) Photochem. Photobiol. 14, 287–305.
[5] Bouges-Bocquet, B. (1973) Biochim. Biophys. Acta 292, 772–785.
[6] Commoner, B. (1961) in: A Symposium on Light and Life (McElroy, W. D. and Glass, B., eds), pp. 356–377, John Hopkins Press, Baltimore.
[7] Amesz, J., Pulles, M. P. J. and Velthuys, B. R. (1973) Biochim. Biophys. Acta 325, 472–482.
[8] Duysens, L. N. M. (1972) in: Proc. 2nd Int. Congr. Photosynthesis Research, Stresa (Forti, G., Avron, M. and Melandri, A., eds), Vol. 1, pp. 19–25, Dr. W. Junk N.V. Publishers, The Hague.
[9] Babcock, G. T. and Sauer, K. (1973) Biochim. Biophys. Acta 325, 483–503.
[10] Esser, A. F. (1974) Photochem. Photobiol. 20, 167–172.
[11] Kok, B., Radmer, R. and Fowler, C. F. (1974) in: Proc. 3rd Int. Congr. Photosynthesis, Rehovot (Avron, M., ed.), in the press, Elsevier, Amsterdam.