Flash Pattern of Oxygen Evolution in Greening Etioplasts of Oat

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Etioplasts isolated from oat leaves that have received two 5 min illumination periods, spaced apart by a 90 min dark interval, are able to evolve oxygen. If this oxygen evolution is measured as consequence of short saturating light flashes a pattern is obtained which in agreement with the Kok-Joliot model shows a damped oscillation with a periodicity of four. As usual for most other systems the maximum flash yield is observed under the third flash. In continuous light such slightly greened etioplasts show a small oxygen gush. From our studies it appears that the second 5 min illumination period is obligatory for oxygen evolution. Omission of this period leads to a condition in which under flashes strictly no oxygen is evolved. In continuous light under these conditions no oxygen gush but rather an uptake is observed after onset of illumination. It appears that in greening etioplasts the flash pattern shows some peculiarities when compared to the normal green conditions. Thus, a flash sequence of a greening etioplast always shows a substantial positive amperometric signal under the first flash. The half time of the state which would belong to this signal is 3 to 4 min in a greening etioplast, isolated from leaves that have received 2 h light. In the course of greening this half time decreases continuously and reaches a normal and constant value of approx. 10 s in chloroplasts from green oat or etiochloroplasts that are prepared from leaves that have received 15 h of light. The result is discussed in terms of metastable S_3 which seems to be a property of the developing oxygen evolving system in greening oat leaves. In the greening system as we use it is shown for the first time that the photoenzyme protochlorophyllide chlorophyllide reductase is DCMU sensitive.

Introduction

Current knowledge and understanding of the oxygen evolving mechanism in photosynthesis is based on the experiments made by Joliot *et al.* [1] and Kok *et al.* [2]. These authors observed that photosynthetic O_2-evolution measured as the consequence of short saturating flashes shows the picture of a damped oscillation with the periodicity of four [1, 2]. This was interpreted in the frame of a model as being due to the successive accumulation of four positive charges (characterized by four S-states) on the donor side of photosystem II which in turn would lead to the oxidation of two molecules of water. This model in particular the "coherent four state Kok Model" [2] was found to account satisfactorily for most of the observations made on algae and higher plant chloroplasts. However, flash sequences of Chlorella and higher plant chloroplasts exhibit an abnormality under the first flash when the flash sequence is mathematically analyzed according to the four state Kok model [3–5]. This abnormality or disturbance disappears if the first flash is excluded from the analysis [4] or if contribution of a more reduced state than S_0, namely S_{-1}, to the initial dark population of S-states is assumed [3, 5]. In agreement with this observation the mathematical fit of any experimental flash sequence is always much better when the five state Kok model [3, 6] is used instead of the traditional four state model. Although, the coherent four state Kok model predicts a very short half life time for S_3 *i.e.* no oxygen under the first flash, a perfect mathematical fit in the four state Kok model always seems to require some substantial oxygen evolution under the first flash [3, 6]. Obviously, this paradox makes flash sequences highly interesting in which a substantial oxygen signal is observed under the first flash. Thus, Lavorel and Seibert [7] have found a large amperometric signal under the first flash in a photosystem II particle preparation from spinach. They attributed this signal to a deterioration of the deactivation mechanism in their particle preparation which in turn would have led to the appearance of metastable S_3 [7]. On the other hand, Bader *et al.* [6] have also observed a substantial signal under the first

Abbreviations: DCMU, N–N′-3,4-dichlorophenyldimethylurea; TES, 2([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino)ethane sulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid.

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flash in preparations from the filamentous blue-green alga *Oscillatoria chalybea.*

Mere interpretation of this result in the four state Kok model would have led to up to 12% metastable S_3 in the S-state population of the dark adapted sample [6]. These authors were able to show that the amperometric signal under the first flash was due to oxygen, but were unable to prove that its origin was water [6].

In the present paper, we describe the properties of the oxygen evolving system in etioplasts in the early stages of greening in oat plants. It appears that we are able to show that the deactivation rate of S_3 is very low at the time of the appearance of the ability to produce oxygen. In the course of greening this deactivation rate progressively increases until a normal situation is reached at greening times around 15–20 h.

Materials and Methods

Etiolated oat plants Avena sativa were grown on soil for seven days in complete darkness et 24 °C. Thereafter, the seedlings were transferred to white light (800 lux) for the indicated lengths of time.

Plastid preparations were obtained from cut-off leaf sections of the etiolated plants. The entire preparation was carried out in green safety light. 80–100 g of leaves were quickly cut to small pieces with scissors and suspended in 300 ml of cold buffer, pH 7.5, containing 20 mM Tes, 20 mM Hepes, 0.5 M sucrose, 1 mM $MgCl_2$, 1 mM EDTA and 0.1% w/v bovine serum albumine. The suspension was blended in a Braun Typ MX 32 blendor under cooling, 5 times for 1 s at high speed followed by 10 s at low speed. The brei was filtered through four layers of cheese cloth. Large cell fragments were separated by a quick centrifugation of 2 min at $500 \times g$. The plastids were sedimented by centrifugation at $1000 \times g$ for 10 min and resuspended in the above described buffer, with serum albumine omitted.

Chlorophyll was determined according to Schmid [8] and *Protein* concentrations were measured according to the method of Bradford [9].

Oxygen measurements were carried out by polarography with the three-electrode-system described by Schmid and Thibault [10] under the conditions specified in the paper by Bader *et al.* [6]. During the measurement, osmotic integrity of the plastids was assured by suspension in the buffer with the above described composition (without serum albumine).

Results

The starting point of the present experiments was the attempt to verify the old experiment by J. H. Smith of 1954 [11] on the onset of the oxygen-evolving capacity in greening plants with our sensitive three-electrode-system [10]. Smith had reported that two short (5 min) preilluminations were sufficient to induce the photoproduction of oxygen in barley leaves, provided these two preilluminations were separated by a dark interval longer than one hour. This old report is in contrast to more recents publication [12–14] where the photoproduction of oxygen in isolated plastids is thought to require greening times of at least 5–6 h.

Figure 1 shows a typical flash pattern of oxygen evolution obtained from plastids isolated from leaves that have received two five minute illumination periods separated by a 90 min dark interval. The experiment confirms Smith and shows the picture of a damped oscillation with a periodicity of four, characteristic of any normal green plant system, with a maximum oxygen production under the third flash. The inset A of Fig. 1 shows that under continuous illumination a distinct oxygen gush is observed which is followed by a small reversible oxygen uptake. The second illumination period of 5 min seems to be obligatory since its omission leads to a condition in which the greening system fails to show any oxygen evolution in the flash light experiment (inset B); continuous light induces in this case no oxygen gush but rather an (reversible) oxygen uptake (inset B). This uptake was not inhibited by the photosystem II inhibitor DCMU nor was it influenced by KCN or catalase.

In the course of the studies on flash sequences of plastids isolated from twice preilluminated leaves (Fig. 1) it was found that the relative oxygen production under the first flash *i.e.* Y_1 of a given sequence was abnormally high when compared to that of normal chloroplasts. In Fig. 2a we have plotted the Y_1 values against the dark time between two successive sequences of 15 flashes. According to Forbush *et al.* [15] the decay of Y_1 measures the decay of S_3. Its half life time was 3 to 4 min in plastids isolated from twice preilluminated leaves whereas the half life time was found to be less than

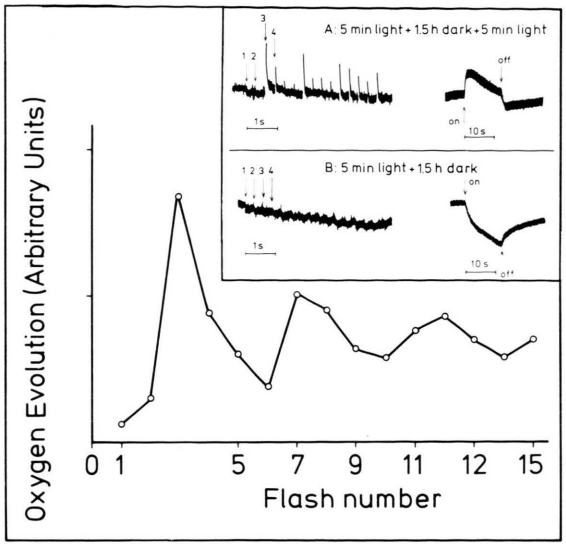

Fig. 1. Oxygen yield pattern obtained from a flash sequence given to dark adapted plastids isolated from plants illuminated by two 5 min periods of continuous white light spaced apart by 1.5 h darkness. Average of five experiments. Inset a) actual oxygen yield pattern of the sequence (left) and oxygen exchange induced by 30 s continuous red light (right) in plastids extracted after the two 5 min illuminations, b) same experimental sequence as a) but with omission of the second 5 min illumination before the plastid isolation.

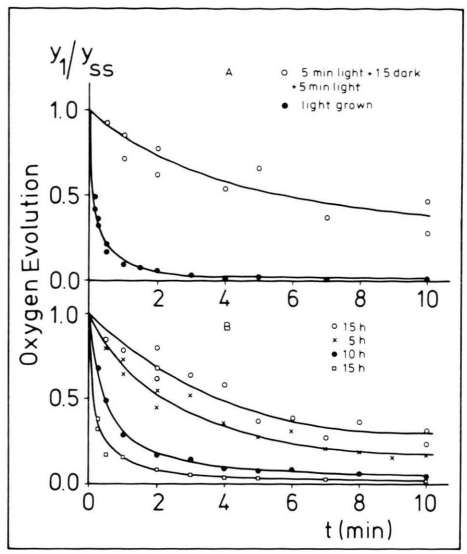

Fig. 2. Oxygen evolution under the first flash (Y_1) as a function of the dark time between two sequences of 15 flashes in plastids isolated at various greening stages: A) (○) etiolated plants illuminated by two 5 min periods spaced by 1.5 h darkness; (●) plants grown for 7 days under white light (14 h light periods); B) etiolated plants illuminated during 1.5 h (○), 5 h (×), 10 h (●) and 15 h (□). The Y_1 values are normalized to the oxygen evolution in the steady-state (Y_{ss}).

Table I. Development of the oxygen-evolving capacity in the course of greening in oat plastids expressed by the increase of the 3rd amperometric signal of a flash sequence.

Greening time	Specific chlorophyll content [μg Chl · mg Protein^{-1}]	Amplitude of the 3rd flash [mV · mg Prot^{-1}]
1	3.9	<0.5
1.5	5.4	6.5
5	23	71
10	57	335
15	88	682

10 s under the same condition in chloroplasts isolated from green oat leaves (Fig. 2 a).

Since a precise chlorophyll determination is difficult in the early greening stages we have referred our amplitude measurement also to unit protein concentration of the plastid preparation. Table I gives with reference to the protein content a rough estimation of the extent of the continuous increase in oxygen evolving capacity in the course of greening, measured as the amplitude of the amperometric signal under the third flash (Y_3).

The dependence of the half life time of S_3 on the greening time is shown in Fig. 2 b. It is seen that the half life time starts out with a high value of 3–4 min at "greening time" 1.5 h. This value decreases progressively with increasing greening times until a low and constant value of 8 to 10 s is reached after 15 h of greening.

Fig. 3 shows a flash pattern of oxygen evolution in dark adapted plastids isolated from leaves that have received 6 h light. It is clearly seen that a distinct positive amperometric signal is observed under the first flash. Sometimes it appears that this signal is masked by a flash induced oxygen uptake, occurring at the same time. In green chloroplasts, isolated under the same conditions, a positive amperometric signal under the first flash was never observed even at high amplification (Fig. 3). The S-state distribution which belongs to the flash sequences of Fig. 4 is shown in Table II. The S-state populations are calculated from a fit in the 4 state Kok model as well as in the 5 state Kok model. It appears that the 4 state Kok model yields a perfect fit if the presence of 6% metastable S_3 in the dark

adapted plastids was acceptable. Clearly, it is also seen that the fit in the 5 state Kok model offers no advantage over the 4 state one.

Figure 4 shows the yield of oxygen in the first four flashes (Y_1 to Y_4) in dependence on the dark time between the flash sequence in plastids from leaves that have been greened during 5 h and in normal green oat chloroplasts. It is clearly seen that the entire deactivation system in the early greening stages is affected. However, although the S_2 and S_1-states decay more slowly than in the green control, the main effect lies on the S_3-state which deactivates about 15 times more slowly than in the green chloroplast.

Fig. 5 shows the correlation between the process of chlorophyll formation on the one hand and development of the photosystem II activity on the other. The upper part of the figure shows that in prothylakoids from etioplasts the photoreduction of protochlorophyllide is DCMU sensitive as shown by the inhibition of the appearance of the 685 nm chlorophyllide fluorescence upon illumination. This is verified by the DCMU inhibition of the appearance of the chlorophyllide peak in the absorption spectrum of prothylakoids from etioplasts (lower part of Fig. 5). The experiment might indicate that a DCMU binding protein is present before the appearance of the ability to evolve oxygen, which,

Table II. S-State population in etioplasts from *Avena sativa* calculated from a fit in the 4-state and 5-state Kok model.

Type of fit	S_{-1}	S_0	S_1	S_2	S_3	Misses α	Double hits γ	Δ%
	(in percent)							
4-State Kok	–	12	77	5	6	14	3.7	3.0
5-State Kok	9	13	66	7	5	12	2.1	2.6

Etioplasts prepared from plants that have received two five minute illuminations spaced apart by a dark interval of 90 min Δ% relative quadratic deviation. Dark adapted for 20 min.

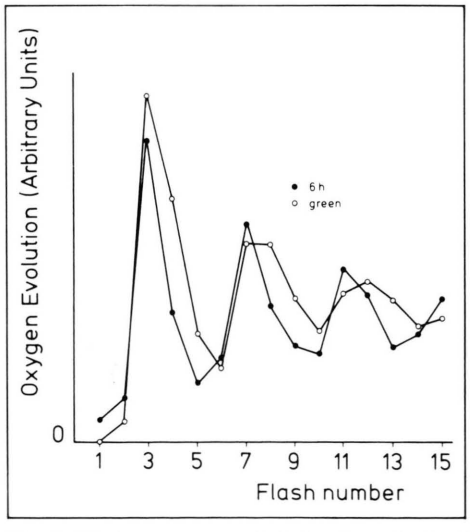

Fig. 3. Flash sequences of dark-adapted plastids isolated from etiolated plants illuminated for 6 h (●) or from light-grown plants (○).

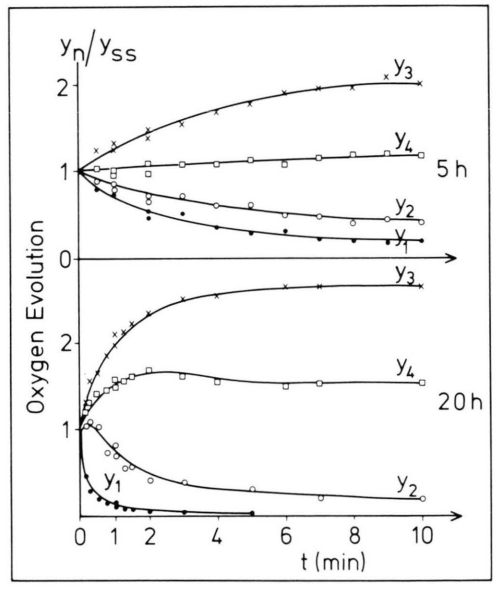

Fig. 4. Oxygen evolution under the first four flashes (Y_{1-4}) of a sequence in dependance on the dark time between two sequences of 15 flashes. Plastids were isolated from etiolated plants illuminated for 5 h (upper part) or 20 h (lower part). The Y_n values are normalized to the oxygen evolution at the steady-state (Y_{ss}).

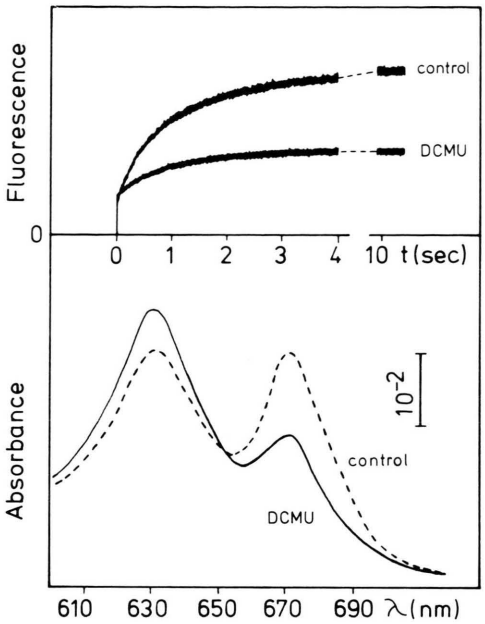

Fig. 5. Effect of 2×10^{-4} M DCMU on protochlorophyllide photoreduction in etioplast membranes, as measured by room temperature 685 nm-fluorescence variations (upper part) and by the absorbance spectra recorded after illumination (lower part).

however, is not operational in the oxygen uptake function shown in Fig. 1, Inset b, but would interact with the process of photoreduction.

Discussion

The present paper contains two principal observations:

The first observation is that the oxygen evolving capacity develops very early in the greening process (see also our previous paper [16]). The observation, as we made it, confirms the experiment by Smith thirty years ago [11] and is at first glance in contrast to reports in which greening times of approx. five hours seemed to be the minimum [12–14]. This, in our opinion, was either due to an insufficient sensitivity of the oxygen detecting devise of the latter authors or due to a loss in plastid integrity. Our observation, namely that two five minutes preilluminations spaced apart by a 90 min dark interval are sufficient to induce the capacity of oxygen evolution (Fig. 1), raises immediately the question whether this early oxygen evolution has the same properties and characteristics as that in a further greened system or in a normal green one. From this point of view it is important to note that if the second 5 min preillumination after the dark period is omitted, an oxygen uptake system is operational (Fig. 1 b) which either is contained in any normal photosynthetic oxygen evolving apparatus (and which becomes detectable under such conditions) or is a precursor function in an early developing stage of the water splitting system. Before answering this question we would like to note that Remy [13] has observed that in early stages of greening in the presence and absence of ferricyanide an uptake rather than an oxygen evolution was observed in the respective plastids. This oxygen uptake was not DCMU sensitive and is at first glace of the type reported by Schmid and Thibault [10]. Moreover, work by Pistorius and Pistorius and Voss [17, 18] has clearly shown that the 49 kD protein in *Anacystis nidulans* which is a constituent of photosystem II has the properties of an arginine oxidase which shows that an oxygen uptake function associated with photosystem II seems to exist. The background of this literature together with our observation that the flash sequence after the second 5 min illumination (Fig. 1) is practically normal, favors the concept that the observed oxygen uptake is functionally very close to oxygen evolution. For instance during the second five minute illumination (Fig. 1 a) manganese might be attached to the 49 kD protein inducing the proper conformation which makes O_2 evolution eventually possible. Without this, light activates in the reaction center a Pistorius type reaction [17] which leads to oxygen uptake. The fact that oxygen uptake in the present greening system and that the uptake reported on by Remy [13] are not sensitive to DCMU gives no problems and is easily explained by the absence of the DCMU binding protein above all since in normal green systems this protein is a rapid turnover protein [19].

The second observation is that in the early stages of greening the decay mechanism of the S-state system seems to be much slower than in normal green chloroplasts. The major effect lies on the S_3-state which decays 30 times more slowly than in the fully greened system. The positive amperometric signal under the first flash implies existence of metastable S_3, provided an artefact can clearly be excluded. The fact that the slow decay time in the early greening stages increases gradually to the normal value (Fig. 4), known from green chloro-

plasts excludes any preparational artefact. In the present case the possibility of a light induced inhibition of an oxygen uptake can be principally excluded for trivial reasons, which was not the case for *Oscillatoria chalybea* preparations [6]. In the cyanobacterium *Oscillatoria chalybea* an interference of respiratory and photosynthetic electron transport, occurring in the same membrane, had to be taken in the account when evaluating such flash patterns. A further artefactual reason for such a positive amperometric signal under the first flash might be the presence of a redox component of the membrane (exposed by the preparation procedure) capable of extracting electrons from the platinum electrode, the half-wave potential of which would be very similar to oxygen itself (Fig. 8 in [6]). Due to such considerations we have recorded flash sequences of *O. chalybea* amonst others in the presence of high amounts of catalase (Bader and Schmid, in preparation). In flash sequences of the cyanobacterium the first signal does not disappear in the presence of catalase. It appears however, that the addition is beneficial to the oscillatory quality of the flash pattern, which means that misses are greatly decreased. In the present case catalase does not abolish the first amperometric signal either nor has KCN any effect on the flash pattern. From this we propose that in early stages of greening or in early evolutionary stage of development such as that represented by the filamentous cyanobacterium *O. chalybea* the S-state system is characterized by a slower deactivation mechanism when compared to Chlorella or normal green chloroplasts. This inherent property becomes especially evident in the S_3-state, where the decay time in the early stages of greening may be by a factor of 30 slower than in the green control.

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