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Analysis of oxygen evolution during photosynthetic induction and in multiple-turnover flashes in sunflower leaves

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Abstract. Exchange of CO_2 and O_2 and chlorophyll fluorescence were measured in the presence of 360 $\mu l \cdot l^{-1}$ CO_2 in nitrogen in *Helianthus annuus* L. leaves which had been preconditioned in the dark or at a photon flux density (PFD) of 24 $\mu mol \cdot m^{-2} \cdot s^{-1}$ either in 21 or 0% O_2. An initial light-dependent O_2 outburst of 6 $\mu mol \cdot m^{-2}$ was measured after aerobic dark incubation. It was attributed to the reduction of electron carriers, predominantly plastoquinone. The maximum initial rate of O_2 evolution at PFD 8000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ was 170 $\mu mol \cdot m^{-2} \cdot s^{-1}$ or about four times the steady CO_2- and light-saturated rate of photosynthesis. Fluorescence measurements showed that the rate was still acceptor-limited. Fast O_2 evolution ceased after electron carriers were reduced in the dark-adapted leaf, but continued for a short time at the lower rate of 62 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in the light-adapted leaf. The data are interpreted to show that enzymes involved in 3-phosphoglycerate reduction are dark-inhibited, but were fully active in low light. In a dark-adapted leaf, respiratory CO_2 evolution continued under nitrogen; it was partially inhibited by illumination. Prolonged exposure of a leaf to anaerobic conditions caused reducing equivalents to accumulate. This was shown by a slowly increasing chlorophyll fluorescence yield which indicated the reduction of the PSII acceptor Q_A in the dark. When the leaf was illuminated, no O_2 evolution was detected from short light pulses, although transient O_2 production was appreciable during longer light pulses. This indicates that an electron donor (pool size about 2–3 e/PSII reaction center) became reduced in the dark and the first photons were used to oxidise this donor instead of water.

Key words: Anaerobiosis – *Helianthus* (photosynthesis) – Photosystem II – Photosynthesis (O_2 evolution) – Respiration

Introduction

In photosynthesis of leaves, reactions of two photosystems, electron transport, carbon reduction and end-product processing are coordinated and regulated depending on external conditions and plant requirements. Relevant studies of these control processes need non-invasive techniques, such as gas exchange and optical measurements. With the rapid-response CO_2-exchange measurement system of Oja (1983), we have studied the light-dependent modulation of carbon-reduction enzymes which occurs within minutes after prolonged darkening of leaves (Siebke et al. 1990). Using a sensitive flow-through oxygen-electrode, we were also able to resolve transients in oxygen evolution which occur within a few seconds after a dark/light transition (Laisk et al. 1989). They were interpreted as a sequence of events which begins with reduction of electron carriers, followed by activation of 3-phosphoglycerate (PGA) reduction (Siebke et al. 1991) and, finally, by a much slower activation of the ribulosebisphosphate (RuBP)-regenerating enzymes.

In the present work we further increase the time-resolution of O_2-evolution measurements using a pulse-differentiation method where O_2 evolution in light pulses of different lengths is measured. This enables us to study O_2 evolution and plastoquinone reduction as determined by the water-oxidizing system and PSII only. Complemented by chlorophyll fluorescence measurements, these data reveal in some detail the control of PSII efficiency by reduction of the primary quinone acceptor Q_A and by processes related to nonphotochemical fluorescence quenching. We present evidence (i) that the maximum capacity of PSII to deliver electrons from water to plastoquinone is at least four times higher than the maximum capacity of carbon reduction and end-

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Abbreviations: Chl = chlorophyll; CRC = carbon reduction cycle; GAPDH = NADP-glyceraldehyde-phosphate dehydrogenase; PFD = photon flux density; PGA = 3-phosphoglycerate; RuBP = ribulose bisphosphate; TCA = tricarboxylic acid cycle

product pathways; (ii) that oxidizing conditions are required not only at the acceptor, but also at the donor side of PSII to support maximum O_2 evolution; (iii) that the photochemical efficiency of PSII is down-regulated when CO_2 uptake into the leaf becomes limiting for photosynthesis.

Materials and methods

Well-fertilized sunflower (*Helianthus annuus* L.) plants were grown in a growth chamber at 800 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 8/16 h 28/22° C day/night in a peat-soil mixture. Attached upper mature leaves of three- to four-week-old plants were used in the experiments.

The custom-built gas-exchange apparatus contains two identical open gas-flow systems in which the gas composition can be adjusted separately (Oja 1983). Both systems (channels) are connected to water-vapour, CO_2 and O_2 analysers. A sandwich-type leaf chamber ($4.4 \cdot 4.4 \cdot 0.3\ cm^3$, gas flow rate $20\ cm^3 \cdot s^{-1}$) can be rapidly switched from the first into the second channel and vice versa. By reducing the volumes, the response time for CO_2 (99% of full deflection) was reduced to 2.3 s with the infrared gas analyser (Infralyt 4; Junkalor, Dessau, Federal Republic of Germany; two 15-cm^3 cuvettes). Oxygen evolution was measured by a silver-lead electrode which was directly exposed to the gas without a covering Teflon membrane (Kiirats 1985). The electrode area was large enough (10 cm of 0.2-mm silver wire around a 5-mm holder stick) to yield the output necessary for a current-recorder which avoids the polarisation of the electrode. The electrode is sensitive to concentration changes only at low partial pressures of O_2 and has a response-time comparable to that of the CO_2-measurement system.

Light pulses were produced from a Xenon-arc lamp (provided with a heat-reflecting mirror) by a shutter. The front of a pulse was 5 ms. When necessary, additional background light was provided by a halogen lamp equipped with heat mirrors.

Chlorophyll fluorescence was measured by means of a PAM 101 chlorophyll fluorometer (H. Walz, Effeltrich, Germany). Fluorescence transients during pulses were recorded by a digital memorizing oscilloscope. Since fluorescence levels changed during the short light pulses, we took a mean value between the maximal and minimal levels recorded during a pulse.

Results

The influence of predarkening time on the dark/light photosynthetic induction of CO_2 and O_2 exchange. In the experiment of Fig. 1A, a *Helianthus* leaf was darkened for different times under aerobic conditions. It was then transferred within 0.5 s to a nitrogen atmosphere which also contained 360 $\mu l \cdot l^{-1}$ CO_2, and light was turned on. At this low concentration the initial CO_2 "gulp" seen at high CO_2 levels, which is caused by stroma alkalization (Oja et al. 1986; Laisk et al. 1989), was scarcely visible. Instead there was a rapid suppression of CO_2 evolution from the tricarboxylic-acid (TCA) cycle in the light in the absence of O_2 (about 60% inhibition). This cannot be caused by reassimilation since the carbon-reduction cycle (CRC) was not yet active and there was no photosynthetic CO_2 uptake (330 and 600 s darkening). The light-sensitive part of the dark CO_2 evolution recovered slowly after the light was switched off (compare dark respiration rates for 30, 60 and 150 s darkening).

The onset rate of CO_2 assimilation depends on the predarkening time. After a short predarkening, enzymes

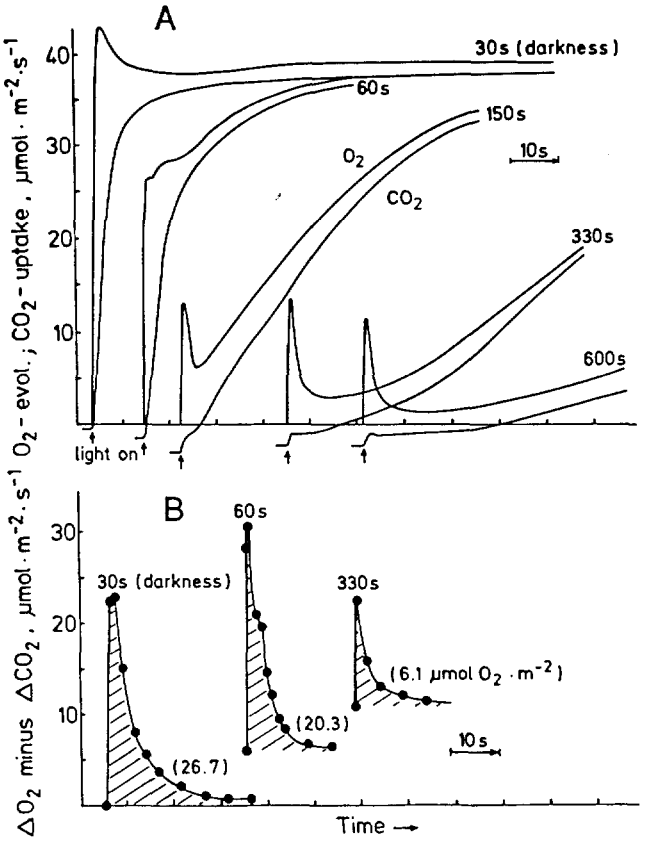

Fig. 1A, B. Dark-light induction of CO_2 uptake and O_2 evolution in a sunflower leaf. **A** Recorded time-courses. Predarkening times in 21% O_2 are shown at the curves; induction was measured at PFD 1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 360 $\mu l \cdot l^{-1}$ CO_2 in N_2. **B** Time-courses of the initial extra O_2 evolution, calculated from the difference between O_2- and CO_2-exchange rates (lasting differences subtracted)

involved in RuBP synthesis are not yet inactivated and the onset rate is determined mainly by activation of PGA reduction (which needs about 10 s, Siebke et al. 1991) and by building up the RuBP pool. Particularly after prolonged darkening, the induction rate for CO_2 uptake indicates slow activation of RuBP-synthesis enzymes.

A monophasic induction of CO_2 uptake was accompanied by an initial burst of O_2 evolution, not detected in the CO_2 uptake. The curves shown in Fig. 1B exhibit time-courses of these O_2 bursts calculated from the difference between the O_2- and CO_2-exchange rates (the O_2-curves in Fig. 1A were shifted downwards to exclude lasting differences between the two rates). Totals of the initial O_2 bursts are represented by the area under these curves.

In Fig. 2 these totals are plotted as functions of the time of predarkening, either in the presence or in the absence of O_2. A sharp decline in the O_2 burst was seen during the initial 150 s of darkening, but then it remained at 6 $\mu mol \cdot m^{-2}$ despite prolonged darkening in 21% O_2. In contrast, when the leaf was predarkened in anaerobiosis, the O_2 burst continued to decline.

Chlorophyll fluorescence was excited and measured during darkening using a modulated red measuring beam of extremely low intensity which did not produce detect-

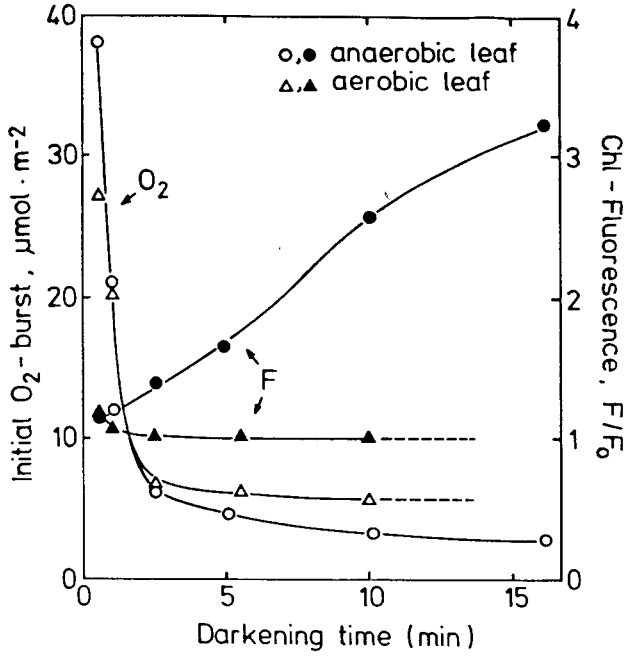

Fig. 2. Totals of O_2 outburst *(open symbols)*, calculated as shown in Fig. 1B, as functions of predarkening time and the presence *(triangles)* or absence *(circles)* of O_2 during predarkening. *F*, chlorophyll fluorescence yield at the end of predarkening, in relation to steady dark fluorescence yield F_o in 21% O_2 *(filled symbols)*

able CO_2 uptake. The fluorescence parameter shown in Fig. 2 is defined as F/F_o where F is the signal at the end of the indicated dark period and F_o is the stable minimal fluorescence yield obtained after extended darkening in the presence of O_2. The maximal value for F/F_o, which was obtained during a short saturating light pulse, was about 5. Variations in F/F_o between 1 and 5 indicate variations in the dark reduction level of the PSII acceptor Q_A. The value of F/F_o was close to 1 in leaves darkened at 21% O_2 indicating that Q_A was highly oxidized. In anaerobically darkened leaves, the level of chlorophyll fluorescence increased with time. Under these conditions, F/F_o was rising slowly and reached 60–70% of its maximum level, indicating considerable reduction of Q_A in the dark under anaerobiosis. The initial O_2 outburst produced by anaerobically darkened leaves decreased below that produced by aerobic leaves. However, it is important to note that even when more than 50% of Q_A was reduced in the dark (as indicated by the F/F_o level), a considerable O_2 burst was still recorded. Upon aeration, the dark fluorescence level and the O_2 yield recovered within a few minutes. Even 0.1% O_2 was remarkably efficient in decreasing F in the dark; 1% O_2 was sufficient for complete oxidation of Q_A in the dark.

Amounts and rates of O_2 evolution during multiple-turnover light pulses. We measured the total amount of O_2 evolved in short light pulses as a function of the duration of pulses. Differentiating the integral against the pulse length enables us to resolve the time-course of O_2-evolution rate. Prior to the measurements, the leaf was adapted either at a low photon fluence density (PFD) of 24 μmol · m^{-2} · s^{-1} at 21% O_2 (in order to partially activate CRC) or in the dark at 21 or 0% O_2.

Figure 3A shows the total O_2 produced during high light (PFD = 913 μmol · m^{-2} · s^{-1}) pulses of increasing duration given to a leaf adapted to low light (open symbols) or to the darkness (closed symbols) in the presence of O_2. Two phases of O_2 evolution can be discerned. From 0 to about 50 ms pulse duration the slopes of the curves are high, which reflects a high O_2-evolution rate. The total yield of this first rapid phase, obtained by extrapolating the second slower phase to t = 0, is about 6 μmol · m^{-2} of O_2. This amount is equal to the initial O_2 outburst in leaves dark-adapted in 21% O_2 (Fig. 2). Using the pulse-differentiation method we could resolve that this initial outburst lasts only 50 ms. During this time the oxidised electron carriers between PSII and the next rate-limiting step become reduced. The pool between these two electron-transport steps is 6 μmol · m^{-2} O_2 = 24 μmol · m^{-2} electrons.

The second phase of O_2 pulse-yields in Fig. 3A characterises the next rate-limiting electron-transport step. After the pulse length exceeds 50 ms, O_2 yield per pulse increased more slowly (light-adapted leaf) or stopped altogether (dark-adapted leaf). This shows that the next rate-limiting electron-transport step is totally inactivated in the dark, but even very low light (PFD = 24 μmol · m^{-2} · s^{-1}) is enough to activate it. From the slope of the curve in Fig. 3A we can calculate that this step has a capacity of 62 μmol · m^{-2} · s^{-1} O_2. This exceeds the maximum light- and CO_2-saturated steady rate of CO_2 assimilation by about 20% (Fig. 4A).

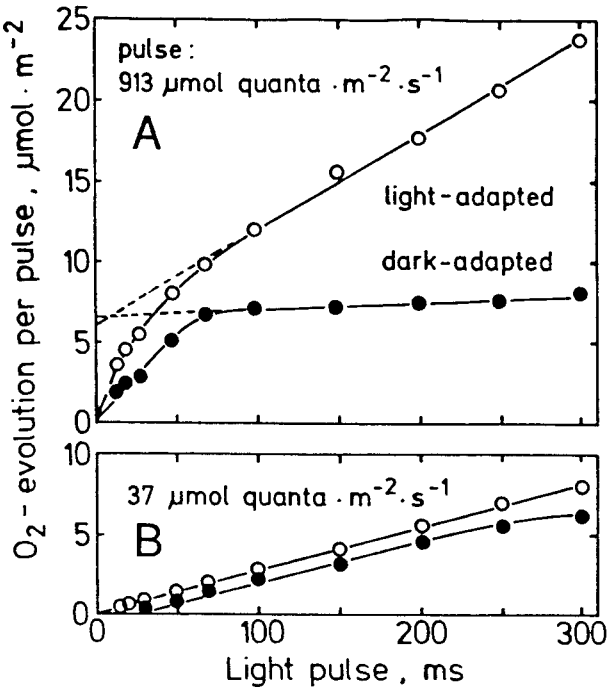

Fig. 3A, B. Total O_2 evolution in light pulses as a function of pulse lenghth at a high pulse PFD (**A**) and a low pulse PFD (**B**). A light-adapted sunflower leaf was pre-exposed at PFD 24 μmol · m^{-2} · s^{-1} and 21% O_2. The O_2 concentration during the dark-adaptation was 21% (**A**) or 0% (**B**)

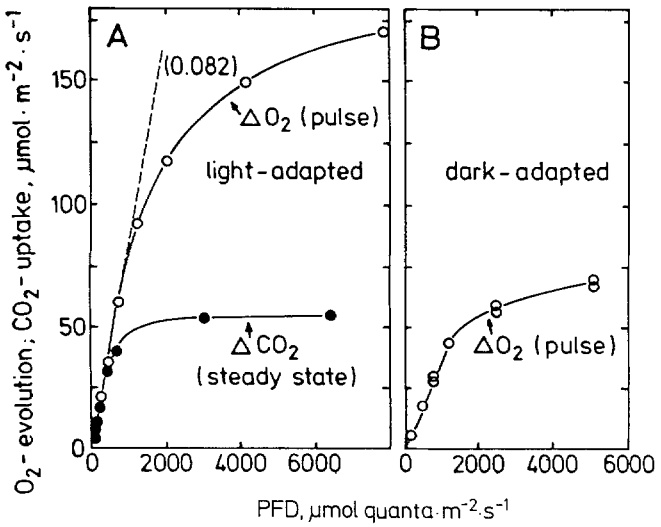

Fig. 4A, B. Initial rate of O_2 evolution at the beginning of illumination as a function of PFD in light-adapted (**A**) and anaerobically dark-adapted (**B**) sunflower leaves. *Filled circles*, steady-state CO_2-uptake rate

Later, a few seconds from the beginning of illumination, O_2 evolution drastically slowed down even in the low-light-adapted leaf (direct recordings in Fig. 1A, also see Laisk et al. 1989). We interprete the second phase in O_2 evolution (with the capacity of 62 μmol · m^{-2} · s^{-1}) as PGA reduction, whilst the later retardation is caused by incomplete activation of RuBP-regeneration enzymes at PFD 24 μmol · m^{-2} · s^{-1} (Leegood et al. 1985; Siebke et al. 1990).

With low-intensity pulses, a linear increase in O_2 evolution with pulse length was seen in the light-adapted leaf (Fig. 3B). About the same slope was obtained with the leaf which had been predarkened in the absence of O_2. However, in the latter case there was a lag of about 25 ms during which no O_2 was detected. This result shows either that photons arriving during that time are dissipated into heat or that they are used for transporting electrons which do not originate from water, but from an alternative donor with a capacity 0.9 μmol · m^{-2} (O_2) = 3.6 μmol · m^{-2} (e).

In the experiment shown in Fig. 4 we studied the initial rate of electron transport by measuring O_2 evolution during short pulses as a function of irradiation density. In order to be sure that the O_2 pulse yield was not lowered as a result of the limited electron-acceptor pool, we used a series of pulse lengths and extrapolated the O_2-evolution rate per pulse to zero pulse length.

A sunflower leaf was first illuminated in an atmosphere of N_2 plus 470 μl · l^{-1} CO_2 with PFD 1000 μmol · m^{-2} · s^{-1} until steady photosynthesis was established. This CO_2 concentration was saturating carbon assimilation, and the high CO_2-uptake rate was stable in the complete absence of O_2 from the ambient gas. The irradiation was then reduced to 20 μmol · m^{-2} · s^{-1} where the quantum yield for assimilation was close to its maximum. Subsequently a series of short light pulses of 15, 30, 50 and 75 ms duration were given. This procedure was repeated using different PFD in pulses. The measured ratio of O_2 evolution/pulse length was extrapolated to zero pulse length to obtain the initial rate.

In Fig. 4A this initial rate is plotted against PFD in the pulses. It saturates at a rate higher than 170, in other experiments even higher than 200 μmol · m^{-2} · s^{-1}, which is similar to the maximum rate observed during the first phase in the experiment of Fig. 3A. It is about four times higher than the maximum steady rate of CO_2 assimilation. Since considerable fluorescence increase was observed during the pulses, the electron transport through PSII under these conditions was limited not by water oxidation, but by plastoquinone reduction. The initial slope of the curve corresponds to a quantum yield of 0.082. The filled symbols in Fig. 4A show the light-response curve of steady CO_2 uptake. At low light the two curves coincide. However, the steady CO_2 uptake saturates at a rate of 50 μmol · m^{-2} · s^{-1}. Obviously, in the plants used here the capacity of the PSII water-splitting complex to deliver electrons to plastoquinone was far higher than the capacity for carbon assimilation.

A similar pulse light-response curve was obtained with leaves predarkened in the presence of O_2 (not shown), while a decline in the initial slope and maximum rate was observed when the leaf was predarkened in the absence of O_2 (Fig. 4B). This may, in part, have been caused by measurement difficulties when the plastoquinone pool was already considerably reduced in the dark, but, as will be shown below, there is a factor which limits oxygen evolution under over-reducing conditions.

Quantum yield of pulse-induced O_2 evolution with open PSII centres. In the steady state, as well as in short light pulses, O_2 evolution can be restricted by the reduction of electron carriers beyond PSII and by accumulation of reduced Q_A. Photosystem-II centres with reduced Q_A are photochemically inactive (closed) and the corresponding chlorophyll fluorescence yield is maximal (F_m). When Q_A is oxidised, the fluorescence yield is at a relatively low and constant level F_o (quencher theory, Duysens and Sweers 1963). The proportion of energy distributed to closed centres (Q_A reduced) can be found from the intermediate fluorescence yield F using linear interpolation between F_o and F_m: $\Phi_F = (F - F_o)/(F_m - F_o)$, and a respective coefficient for fluorescence quenching $q_P = 1 - \Phi_F$ can be taken as the fractional coefficient for energy distribution to open centres (Schreiber et al. 1986).

During light pulses we recorded fluorescence and O_2 evolution simultaneously. Figure 5A shows Φ_F in 50-ms pulses as a function of pulse PFD for differently pretreated leaves; Φ_F indicates accumulation of Q_A^-. It should be noted, however, that because of energy exchange between PSII units $F_v = F - F_o$ is not linearly related to Q_{A-}. The increase in F_v was highest in an anaerobically dark-adapted leaf. It was much smaller in an anaerobically light-adapted leaf and the least in an aerobically dark-adapted leaf. The data clearly indicate appreciable restriction of electron transport from the acceptor side of PSII, particularly in anaerobically dark-adapted leaves.

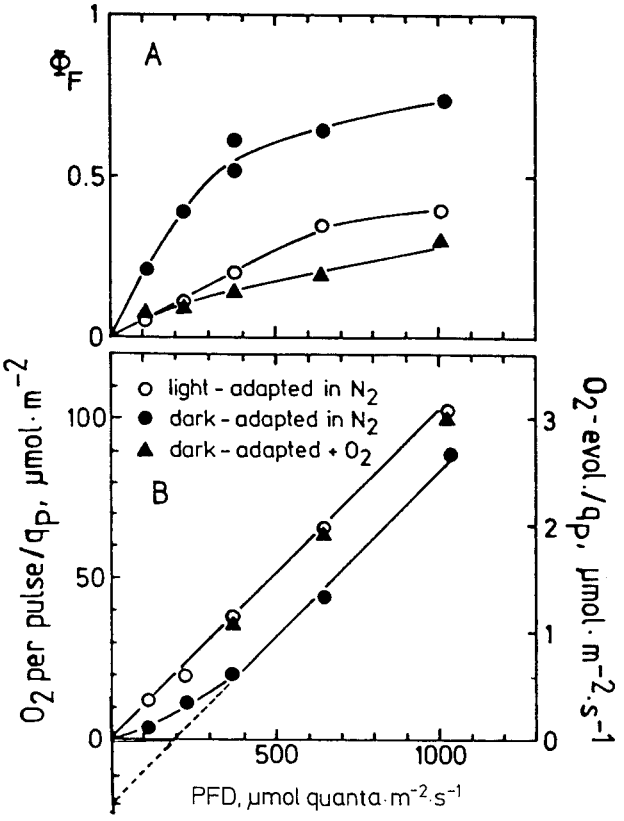

Fig. 5A, B. Fluorescence yield and oxygen evolution of a sunflower leaf subjected to 50-ms light pulses. **A** Average proportion of photons (Φ_F) delivered to PSII centres with reduced acceptor Q_A in 50 ms pulses of different PFD, calculated from chlorophyll fluorescence data. **B** Oxygen evolution per 50-ms pulse *(left ordinate)* and average O_2-evolution rate during the pulse *(right ordinate)* with 'open' (Q_A oxidized) PSII centres, calculated by dividing the measured O_2 evolution by $q_P = 1 - \Phi_F$ from **A**. Different symbols stand for different pretreatments

Following a procedure proposed by Weis and Berry (1987), we divided the O_2-evolution rates actually measured by q_P in order to correct for the restriction by reduced Q_A and to arrive at the possible O_2-evolution rate if all centres had been oxidised (open). As a result of this correction the saturating curves in Fig. 5A converted into linear relationships (Fig. 5B), showing that the restriction of electron transport was completely on the acceptor side when short light pulses were given. In leaves adapted to low light or darkened in 21% O_2, the slope of the light curves increased to 0.103, which is similar to the quantum yield in many C_3 species (Björkman and Demmig 1987). A very similar slope was obtained with the anaerobically predarkened leaf at higher pulse PFDs. However, with this leaf the curve was concave. The linear part of the curve extrapolated to $-0.6\ \mu mol \cdot m^{-2}$ (O_2) = $2.4\ \mu mol \cdot m^{-2}$ (e). It roughly agrees with the experiment of Fig. 3B, showing that, in an anaerobically predarkened leaf, the first quanta absorbed by PSII are transporting electrons which do not originate from water. Only after that is light used as efficiently for water splitting as in light-adapted or aerobically dark-adapted leaves.

Pulse-induced O_2 evolution during steady-state photosynthesis. In the above experiments we used either darkness or very low light for the pretreatment of leaves. In the following experiment we study pulse-induced extra O_2 evolution on top of steady-state rates at higher PFDs.

A leaf was kept in an atmosphere containing 1% O_2 and $500\ \mu l \cdot l^{-1}$ CO_2 and illuminated for at least 15 min with different PFDs (0 to $300\ \mu mol \cdot m^{-2} \cdot s^{-1}$). At these limiting PFDs the level of fluorescence during steady-state assimilation was very close to F_o, as already reported (Weis and Berry 1987). This shows a low steady-state reduction level of Q_A (Dietz et al. 1986). Then, a series of 50-ms pulses of different PFDs was given on top of the background light and the pulse-induced extra O_2 evolution and fluorescence were recorded. The potential rate of O_2 evolution during the pulses was calculated as in Fig. 5B.

The light-dependence of the potential O_2-evolution rate (corrected for Q_A reduction) was linear regardless of the background light. It had a slope of 0.089 O_2 per quantum (Fig. 6). When the CO_2 concentration was lowered to $100\ \mu l \cdot l^{-1}$ at a PFD of $300\ \mu mol \cdot m^{-2} \cdot s^{-1}$ the steady rate dropped because photosynthesis was limited by CO_2 and light was in excess. After the leaf had adapted to this condition the slope of the plot of O_2 evolution vs. pulse PFD was reduced to 0.056, while the maximal level of variable fluorescence was lowered by about 60% (not shown). Both, the O_2 quantum yield and fluorescence yield recovered within 5–10 min after the leaves were allowed to re-adapt to low light or high CO_2.

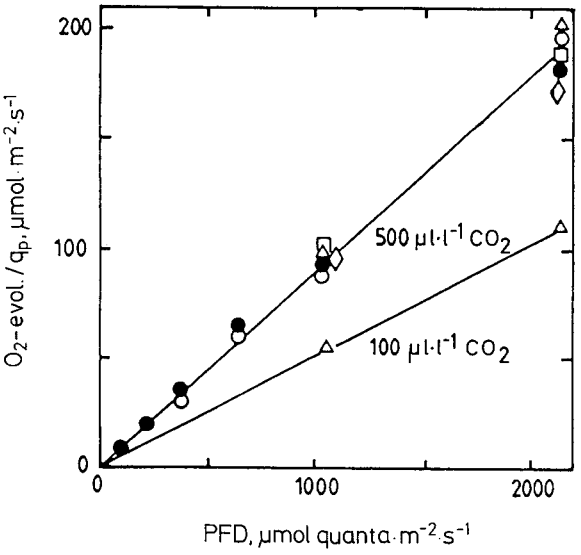

Fig. 6. Pulse-induced extra O_2 evolution from 'open' PSII centres as a function of pulse PFD and CO_2 concentration. The sunflower leaf was preadapted at different PFDs from 0 to $300\ \mu mol \cdot m^{-2} \cdot s^{-1}$ at $500\ \mu l \cdot l^{-1}$ CO_2 in 1% O_2. Then a series of 50-ms pulses of different PFDs was given, extra O_2 evolution and fluorescence were measured, and O_2 evolution divided by $q_P = 1 - \Phi_F$. Different symbols stand for different preadaptation PFDs ($\mu mol \cdot m^{-2} \cdot s^{-1}$): ○, 40; ◇, 80; □, 160; △, 300; ●, darkness. At the preconditioning PFD $300\ \mu mol \cdot m^{-2} \cdot s^{-1}$, CO_2 concentration was lowered to $100\ \mu l \cdot l^{-1}$ and the measurements were repeated

We relate this reversible decline in the potential photochemical yield of PSII to a control mechanism as suggested by Weis and Berry (1987): when photosynthesis is limited by biochemical reactions and light is in excess, a high proton gradient is built up and it feeds back to PSII, converting it into a low-efficiency form which quenches part of the quanta via a nonphotochemical and radiationless route (see review by Krause and Weis 1991).

Discussion

In this work we have continued our studies of homeostatic regulation and internal limitation of photosynthesis in intact leaves using gas-exchange and optical methods. Applying the pulse-differentiation method, time-resolution in O_2-evolution measurements could be increased into the millisecond region.

Using different dark exposures we analyzed the sequence and speed of dark/light modulation of sequential rate-controlling steps in photosynthesis. When light was switched on after a short predarkening period (30–60 s), rapid O_2 evolution started immediately (Fig. 1). There was a great excess of O_2 evolution over CO_2 uptake during the first 10–20 s, altogether up to 30–40 $\mu mol \cdot m^{-2}$. Evidently, during a short period of darkening the CRC enzymes remained active and electrons were consumed for PGA reduction and building up the pools of CRC intermediates. A recent study of NADP-glyceraldehydephosphate dehydrogenase (GAPDH) modulation using transient overreduction of P700 as an indicator of the restricted electron transport (Siebke et al. 1991) showed that the activity of this enzyme changes much faster than that of other light-regulated CRC enzymes. Therefore, photosynthetic induction after 30 and 60 s darkening includes GAPDH activation. The slower induction of CO_2 uptake after longer predarkening is then caused by slower dark/light modulation of the RuBP-regeneration enzymes. The different speeds of modulation of GAPDH- and RuBP-regeneration enzymes is interesting since all the CRC enzymes are known to be activated by the same thioredoxin (thioredoxin f; Buchanan 1980; Scheibe 1990; Knaff and Hirasawa 1991). The rate of O_2 evolution exceeds that of CO_2 uptake not only in the initial phase of induction, but also during the slow induction of RuBP regeneration. Finally, a steady difference, about 1.5 $\mu mol \cdot m^{-2} \cdot s^{-1}$, or 4% of the maximum photosynthetic rate, remains. This extra O_2 evolution indicates the rate of nitrite reduction and other reducing reactions which proceed in parallel with carbon reduction. The corresponding linear electron transport may be important for building up a high proton gradient, especially at the beginning of photosynthetic induction when the O_2/CO_2 ratio is much greater than 1.

The effect of light on respiratory CO_2 evolution has been the subject of long-lasting discussions (Laisk 1977; Graham 1980). The experiment of Fig. 1 shows that respiratory CO_2 evolution is rapidly suppressed in the light in the absence of CO_2 uptake and under anaerobiosis. When O_2 was withdrawn in the dark, CO_2 evolution continued undisturbed for about 30 min before it slowly started to decline (data not shown). It is known that CO_2 evolution from the TCA cycle does not stop in the absence of O_2, provided that reducing equivalents can be diverted to other acceptors (Graham 1980). During photosynthetic induction, electron pressure was high and, in the absence of O_2 as electron acceptor, the NADH/NAD ratio might considerably increase in the cytosol and, via the malate translocator, also in the mitochondria (Heldt et al. 1990; Heineke et al. 1991). Therefore, this experiment confirms that CO_2 evolution from the TCA cycle is controlled by reducing conditions in the mitochondria (Wiskich and Dry 1985).

In our experiments, when the leaves were kept in the dark under anaerobiosis, respiratory CO_2 evolution continued. As a result of that, reducing equivalents accumulated in the mitochondria. They were equilibrated with the cytosol and with chloroplasts. This caused the slow reduction of Q_A in the dark and accumulation of reduced electron carriers (Fig. 2). Among other electron carriers, a donor which can efficiently compete with the water-splitting complex becomes reduced. Our leaves contained 550 $mg \cdot m^{-2}$ Chl. Assuming 500 molecules of chlorophyll per PSII (Graan and Ort 1984), we have 1.22 $\mu mol \cdot m^{-2}$ PSII centres. The measured alternative donor pool was 2.4–3.6 $\mu mol \cdot m^{-2}$. Therefore, the first two to three photons absorbed by PSII transfer electrons supplied by this donor, and normal water-splitting begins only after that (Fig. 5). A possible mediator for electron donation is cytochrome b_{559}, with two heme molecules per PSII (Shuvalov et al. 1989; Hansson and Wydrzinsky 1990), which is known to be capable of reducing the oxidized reaction centre even at the temperature of liquid nitrogen (Boardman et al. 1971). It can also catalyze cyclic electron flow around PSII (Heber et al. 1979). When the leaf was preconditioned in anaerobiosis, even low light prevented oxidation of this alternative donor. We do not wish to emphasize here the importance of this alternative donor as a mediator of cyclic electron transfer around PSII. Rather, this experiment demonstrates how tightly the redox systems of mitochondria, cytosol and chloroplasts are interconnected. A period of just 10–20 min of anaerobiosis in the dark is sufficient to considerably increase the reduction of the electron-transport chain in chloroplasts.

In extended darkness, all light-regulated CRC enzymes are inactivated. Under these conditions the initial O_2 burst which remains (Figs. 1, 2) reflects the pools of available electron-transport intermediates, such as plastoquinone and ferredoxin. As long as ferredoxin-NADP reductase is active, NADP is also included. The measured pool was about 6 $\mu mol \cdot m^{-2}$ (O_2) = $\mu mol \cdot m^{-2}$ (e) = 60 nmol (e) $\cdot$ $(mg\ Chl)^{-1}$. The capacity of electron uptake by the chloroplast plastoquinone pool may be as high as 100 $nmol \cdot (mg\ Chl)^{-1}$ (Crane et al. 1966). On the other hand, Graan and Ort (1984) have determined that there are six plastoquinone molecules per PSII centre in spinach leaves which, in our case, could accept only about 15 $\mu mol \cdot m^{-2}$ (e). Under anaerobiosis, when Q_A was already considerably reduced, not all of these intermediates were completely reduced, since the

initial O_2 burst declined but did not disappear (Fig. 2). As plastoquinone should be completely reduced under these conditions, the observation indicates that other carriers besides plastoquinone were also available during the initial O_2 burst.

Using the pulse-differentiation method, we could resolve the events which take place in O_2 evolution during the first 300 ms of light. The electron-transport intermediates become reduced already within the first 50 ms. The electron-donation capacity of PSII is very high. In the dark-adapted leaf, further electron transport was completely blocked (Fig. 3), probably on the route from PSI to PGA (Siebke et al. 1991). The very low PFD of 24 $\mu mol \cdot m^{-2} \cdot s^{-1}$ was sufficient to remove the block and to support an electron-transport rate of 62 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in O_2 equivalents (Fig. 3). This somewhat exceeded the steady-state CO_2- and light-saturated rate of photosynthesis. At the same time it was clear that the RuBP-regeneration chain was activated only partially (Fig. 4 in Siebke et al. 1990), far less than necessary to support the maximum rate of photosynthesis. This once again emphasizes the specific modulation characteristics of GAPDH compared with other CRC enzymes. Actually, it is possible that plastoquinone oxidation rather than GAPDH was limiting during the second phase of electron-transport induction (Fig. 3). This electron-transport step has an important control function (Siggel 1974; Weis et al. 1987) and its rate may, therefore, be close to the maximum steady-state photosynthetic rate, as observed in our experiment of Fig. 4.

With the pulse-differentiation method we were able to measure the light curves of PSII since they are determined only by electron donation from water and plastoquinone reduction. As expected, the quantum yield of steady-state photosynthesis is close to that of the PSII complex. However, the maximum rate of plastoquinone reduction by PSII exceeds the maximum steady-state photosynthesis by a factor of about four (Fig. 4). Fluorescence analysis showed that this maximum rate is limited by plastoquinone reduction, not by water oxidation. This step involves the diffusion of oxidized plastoquinone to the Q_B site of PSII, its binding to the site, electron transport from Q_A to Q_B and unbinding of reduced plastoquinol. These processes together, including plastoquinone diffusion, are too rapid to be even close to being rate-limiting when PSII centres are in an active state. These data support the view that plastoquinone diffusion is not rate-limiting in photosynthesis (Ort 1986).

When the pulse experiments where done against a background of different irradiances, the PSII maximum rate remained high as long as carbon assimilation was not limiting the overall rate. When the CO_2 concentration was decreased and carbon assimilation became limiting, the PSII quantum yield, as calculated from the O_2 pulses, also decreased. The intrathylakoid pH-dependent control of PSII efficiency has been repeatedly demonstrated in steady-state experiments with leaves (Weis and Berry 1987; Genty et al. 1989; reviewed by Krause and Weis 1991) and in isolated PSII particles (Krieger and Weis 1990). In Fig. 6 its existence is demonstrated from measurements of electron transport through the PSII complex in intact leaves.

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