Oxygen Uptake during Photosynthesis of Isolated Pea Chloroplasts

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Z. Naturforsch. **54c,** 209–219 (1999); received October 5/November 13, 1998

Pisum sativum, Chlororespiration, ^{18}Oxygen Consumption, Photophosphorylation, Intact Isolated Chloroplasts

Mass spectrometric analysis of the gas exchange of illuminated leaflets of 10–14 d old pea seedlings revealed not only $^{16}O_2$-liberation from photosynthetic $H_2{}^{16}O$-splitting, but also uptake of $^{18}O_2$, applied to the gas phase of the reaction vessel. Isolated intact chloroplasts of such leaflets suspended in a medium containing $NaHCO_3$ and glycerate 3-phosphate, on irradiation with blue (λ 448 nm) or red (λ 679 nm) light also produced $^{16}O_2$ from water oxidation and consumed $^{18}O_2$ from the gas phase. The two reactions were saturated at the same quantum fluence rates. Uptake of ^{18}oxygen was not affected by inhibitors of mitochondrial respiration (alternative pathway included), such as rotenone (5×10^{-5} M), antimycin A (5×10^{-6} M), KCN (10^{-3} M), SHAM (10^{-3} M), or propylgallate (10^{-3} M). It was, however, absent, when photosynthetic ^{16}oxygen evolution was completely inhibited by DCMU (10^{-5} M). DBMIB (10^{-5} M), assumed to prevent electron flow from plastoquinone pool to the cytochrome b_6/f-complex, suppressed photosynthetic oxygen evolution, but did not impair uptake of $^{18}O_2$. A similar result was obtained at application of 4×10^{-5} M antimycin A.

The data are interpreted to show a drain off to molecular oxygen of light-excited electrons from the photosynthetic electron transport chain at the site of plastoquinone pool during photosynthesis. This corresponds to chlororespiration, originally described for *Chlamydomonas* in darkness by Bennoun (1982). It is discussed, whether O_2-uptake during photosynthesis is an additional means for providing ATP for photosynthetic CO_2-reduction by increasing the proton gradient across the thylakoid membrane.

Introduction

Influences of light on respiratory processes in plants, called "Lichtatmung" have been assumed and discussed for more than a century (*e.g.* Rosenstock and Ried, 1960). Originally confined to influences of visible radiation on mitochondrial respiration, ideas on comparable reactions in chloroplasts came up with time (see *e.g.* Goedheer, 1963). Especially the hypothesis, eukaryotic cell organells might originate in unicellular prokaryotic organisms substantiated these ideas considerably. Photosynthetic bacteria, proposed to be tentative candidates for chloroplasts, are provided with photosynthetic and respiratory electron transport chains on the same intracellular membranes. Both chains have components in common which leads to pronounced interdependences (*e.g.* Peschek, 1983, 1987; Scherer *et al.,* 1988; Vermaas *et al.,* 1994). In 1982 finally, Bennoun developed a detailed concept for respiratory activity in chloroplasts. Mainly from his experiments on fluorescence in flash light exposed *Chlamydomonas* cells and from the proof of NAD(P)H-plastoquinone oxidoreductase activity in thylakoids of this alga (Godde and Trebst, 1980; Godde, 1982), he proposed an electron pathway in the thylakoid membrane leading from NAD(P)H to molecular oxygen, involving the plastoquinone pool of the photosynthetic electron transport chain between photosystem II and photosystem I. He designated this process "chlororespiration" and assumed it to be responsible for recycling of NAD(P)H and ATP resulting from starch degradation in the dark. From the following extensions and modifications of this concept (Peltier *et al.,* 1987; Willeford *et al.,* 1989; Bennoun, 1994; Endo and Asada, 1996), also genetic clues to existence of chloroplast gene products (frxB = ndh I) comparable with those of the respiratory complex I of mitochondria shall be

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropanyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; SHAM, salicylhydroxamic acid.

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0939–5075/99/0300–0209 $ 06.00

mentioned here (Wu *et al.,* 1989; Zhang and Wu, 1993; Matsubara *et al.,* 1995). With time, indications for existence of chlororespiration have been reported also for some other unicellular algae, such as *Chlorella* (Bennoun, 1982; Chemeris *et al.,* 1996), *Pleurochloris* (Buechel and Garab, 1995), *Mantoniella* (Wilhelm and Duval, 1990) and *Phaeodactylum* (Caron *et al.,* 1987; Ting and Owens, 1993).

Whether chlororespiration-like processes exist also in chloroplasts of higher plants is not clear yet. Experimental indications from physiological and biochemical data are rather scarce. There are indirect evidences from fluorescence studies with leaves, protoplasts and open cell preparations of tobacco (*Nicotiana tabacum*) and of isolated intact pea (*Pisum sativum*) chloroplasts (Garab *et al.,* 1989) as well as from maize (*Zea mays*) leaves (Damdinsuren *et al.,* 1995). Dark reduction of the photosynthetic plastoquinone pool has been observed in *Amaranthus* leaves (Groom *et al.,* 1993). But there are only a few – additionally not completely unequivocal – results from measurements with isotopes allowing discrimination between oxygen produced and oxygen consumed by chloroplasts of tobacco (Gruszecki *et al.,* 1994). Beside these, there are some genetic data which indicate the existence of this pathway also in higher plants. Comparable with the findings for *Chlamydomonas* mentioned above, genes for components of NADH dehydrogenase, *i.e.* of complex I in the mitochondrial respiratory chain (= ndh genes), have been identified in the genome of chloroplasts of tobacco (*Nicotiana tabacum*), of maize (*Zea mays*), of wheat (*Triticum vulgare*), of broad beans (*Vicia faba*), of sugar beet (*Beta vulgaris)* and of rice (*Oryza sativa*) (Meng *et al.,* 1986; Fearnley *et al.,* 1989; Arizmendi *et al.,* 1992; see also Matsubara *et al.,* 1995).

In this paper, we report on oxygen consumption of isolated intact chloroplasts of *Pisum sativum* during photosynthesis with blue or red light of various quantum flux densities. The data from mass spectrometric analyses are interpreted to show existence of an electron transport from photosystem II to molecular oxygen via the photosynthetic plastoquinone pool, *i.e.* chlororespiration-like activity in a higher plant chloroplast.

Materials and Methods

All investigations were performed with leaflets or leaflet chloroplasts of *Pisum sativum* LINNÉ, Kleine Rheinländerin. Plants were grown on Perlite (Agriperl, Perlite-Dämmstoffe GmbH & Co, Dortmund/Germany) from the seeds in a growth chamber under a 13 h light/11 h dark regime at 25 °C/18 °C. Light intensity was 8000 lux. Relative humidity was 55–60% throughout. For measurements, 12–14 d old plants were used. They were about 10 cm high and had developed 4 leaves.

Preparation of intact chloroplasts

The method of Cerovic and Plesnicar (1984), (see also: Walker, 1980) was used, in principle. 80 g pea leaflets were disintegrated with 300 ml of half-frozen 2 mM HEPES-buffer pH 7.8 plus 343 mM sorbitol, 0.4 mM KCl, 0.04 mM EDTA in a blender at 4 °C. The homogenate was filtered through 10 layers of cheese cloth and centrifuged for 20 s at 2600×*g* plus 80 s at 2000×*g*. The resulting pellet was resuspended with a fine hair brush in 0.5 ml 50 mM HEPES-buffer pH 7.9 plus 330 mM sorbitol, 10 mM KCl, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.2% RSA. The chloroplast suspension obtained was purified by centrifugation for 10 min at 4000×*g* of 2 ml on a Percoll gradient (Percoll in HEPES-buffer pH 7.9 plus 330 mM sorbitol, 10 mM KCl, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.2% RSA, 50/50 v/v) prepared before by centrifugation for 100 min at 10000×*g* (Mourioux and Douce, 1981). The band of intact chloroplasts was pipetted to 40 ml of the above HEPES-buffer in a test tube. Chloroplast were liberated from adhering Percoll by gentle shaking and following centrifugation for 2 min at 3300×*g*. After resuspending them in 1 ml of the above buffer, they were ready for analyses. Their intactness was tested by comparing $K_3[Fe(CN)_6]$-dependent O_2-evolution before and after an osmotic shock. Osmotic breakage of the chloroplasts allows photochemical reduction of the molecule which cannot pass the envelope (Lilley *et al.,* 1975). For experiments, only preparations with at least 90% of intact chloroplasts were used.

Oxygen exchange measurements

Polarography

A Clark type oxygen electrode (Rank Brothers, Cambridge/England) was used. It was mounted in the wall of a plexiglass reaction chamber (3×3×3 cm) placed in a larger plexiglass vessel through which water of 25 °C was pumped continuously. The above prepared chloroplast suspension was diluted to a chlorophyll concentration of 20 ± 0.5 µg/ml by adding 40–80 µl to 2 ml 50 mM HEPES-buffer pH 7.9 plus 330 mM sorbitol, 10 mM KCl, 1 mM EDTA, 5 mM $Na_4P_2O_7$, 0.15 mM KH_2PO_4, 1 mM ATP, 10 mM $NaHCO_3$, 0.5 mM glycerate 3-phosphate. After filling with this mixture, the reaction chamber was closed air-tight and the chloroplast suspension stirred gently with a magnetic stirrer. Voltage applied to the electrode was 800 mV. For illumination, a projector Leitz Prado 250 W was used. Blue light was obtained by passing its radiation through plexiglass filter nr. 627 (3 mm) and a cuvette with $CuSO_4$ ($E_{\lambda 623\,nm} = 0.7$), red light by using plexiglass filter nr. 501 (3 mm), both from Röhm GmbH, Darmstadt/Germany. Quantum fluence rates were determined with quantum spectrometer QSM 11-2500 (Techtum Instruments, Sweden).

Mass spectrometry

Oxygen exchange measurements were carried out with the Stable Isotope Ratio Mass Spectrometer "delta" from Finnigan MAT (Bremen/Germany). The device operated with a two directional focussing Nier type ion source. The ion source (tungsten filament) was operated at 140 °C and had a high stability regulation of electron emission. Fast responses of the detector to changes in the partial pressures of the gas phase were achieved by direct connection of the reaction chamber to the main vacuum of the ion source, bypassing the usual inlet system (Bader *et al.*, 1987). Detection and recording of the oxygen signals $m/e = 32$, $m/e = 36$ were performed simultaneously. The reaction chamber used was a home-made massive steel cell with a plexiglass screw cap whose total internal volume of 19 ml was separated from the gas phase of the ion source by a teflon membrane at the bottom of the chamber. The total membrane spanned surface was 12 cm^2. Into this chamber were given either 2 ml of the diluted chloroplast suspension or 2 ml of the buffer used for diluting the chloroplast suspension plus an intact leaflet. The liquid phase in the reaction chamber was uniformly 1.7 mm high. Chloroplasts were left settling down for 45 min during which time they formed a monolayer on the teflon membrane at the bottom. The small leaflet (the third oldest was always selected) was usually placed upside up on the buffer. There was no significant difference in response when placing it upside down or submerging it in the buffer.

For illumination, Leitz Prado 250 W projector was used. Blue and red light of narrow spectral regions were obtained with interference filters AL λ 448 nm, halfwidth 18 nm, T_{max} = 50% and AL λ 679 nm, halfwidth 14 nm, T_{max} = 45% (Schott & Gen., Mainz/Germany).

Because of the vacuum applied, the reaction chamber could not be closed air-tight; sucked off gases had to be replaced by the outer atmosphere. Calibration performed with oxygen liberated by catalase from H_2O_2 in the reaction chamber will therefore be not absolutely correct. In addition, in case of leaflet or chloroplast analyses additional and different diffusion processes must be expected. Nevertheless, the data obtained by these calibrations did not deviate by more than 10% from those of polarometric determinations in a closed system. We, therefore, present them in the following, indicate however their approximate character. Different from this, the ratios of the responses of each sample should be correct without limitations. For further technical details see Bader and Schmid (1989), Bader *et al.* (1987, 1992).

Chemicals

Antimycin A and propylgallate were purchased from Merck, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), rotenone and salicylhydroxamic acid (SHAM) from Sigma. All substances were dissolved in pure ethanol. Their final concentrations are given in Results and Discussion. Application of ethanol at the respective concentration had no measurable influence on the response examined.

Results and Discussion

Oxygen exchange of intact pea leaflets

When young leaflets (∅ about 2 cm) of 12 d old *Pisum sativum* seedlings were placed on HEPES buffer prepared with $H_2{}^{16}O$ and containing $NaHCO_3$ in a chamber with $^{16}O_2$ and $^{18}O_2$ in the gas phase, $^{16}O_2$-uptake in darkness and $^{16}O_2$-evolution in the light were clearly measurable by mass spectrometry (Fig. 1). Oxygen-evolution from photosynthetic water splitting was comparably high in blue and in red light of equal quantum fluence rates. In darkness, also $^{18}O_2$ decreased. This was, however, not – or at least not completely – dependent on metabolic reactions, since a comparable decrease was also observed in controls lacking any living material (Fig. 1, inset). It resulted from sucking in into the vacuum of the measuring device of $^{18}O_2$ from the limited amount applied to the reaction chamber. In contrast, $^{16}O_2$ lost by this means could continuously be replaced from the atmosphere. The decrease in $^{18}O_2$ became, however, up to 10 times larger when the leaflets were exposed to light. Clearly, light enhanced – or possibly induced new – oxygen consuming reactions in the young pea leaflet. Short and long wavelength visible radiation yielded the same result. Fig. 1 shows original recordings of an exemplary experiment with very pronounced responses. (Note, that concentrations of $^{16}O_2$ and of $^{18}O_2$ have been recorded at different sensitivities of the recorder; alterations in $^{18}O_2$-concentrations are thereby larger by factor 4!) All leaflets tested reacted likewise, in general, whereas the extent of the responses varied largely.

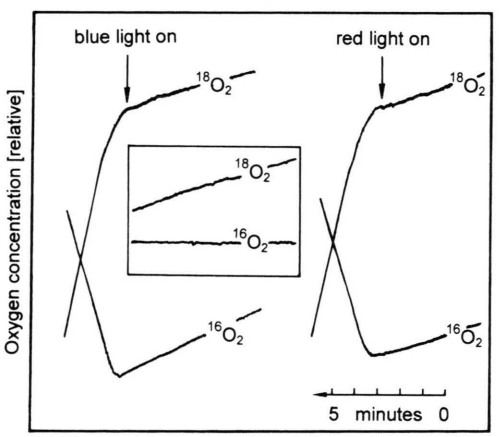

Fig. 1. Oxygen exchange of young leaflets of *Pisum sativum* in darkness and under blue (λ 448 nm) or red (λ 679 nm) light of equal quantum fluence rates of 30 μmol·m^{-2}·s^{-1} determined by mass spectrometry. Leaflets were placed on buffer (2 ml) made up with $H_2{}^{16}O$ (composition see Materials and Methods) in a reaction chamber in which $^{16}O_2$ of the natural gas phase was partly substituted by $^{18}O_2$. Both isotopes were brought to equilibrium with the aqueous phase. Recording sensitivity was 50 mV for $^{18}O_2$ and 200 mV for $^{16}O_2$. $^{16}O_2$-evolution: blue light = approx. 28.4 μmol·mg chlorophyll^{-1}·h^{-1}, red light = approx. 25.6 μmol·mg chlorophyll^{-1}·h^{-1}, $^{16}O_2$-consumption: blue light = approx. 3.9 μmol·mg chlorophyll^{-1}·h^{-1}, red light = approx. 2.1 μmol·mg chlorophyll^{-1}·h^{-1}, $^{18}O_2$-consumption: blue light and red light = approx. 8.9 μmol·mg chlorophyll^{-1}·h^{-1}. Inset: Mass spectrometric determination of $^{16}O_2$ and of $^{18}O_2$ applied to the gas phase over plain phosphate buffer. Original recordings.

Oxygen exchange of isolated chloroplasts

To localize the light-enhanced oxygen uptake of the intact leaflet, isolated intact chloroplasts were analyzed comparatively. Using a suspension medium containing $NaHCO_3$ and glycerate 3-phosphate, polarometric analysis of such preparations revealed no oxygen uptake in initial darkness. It yielded however pronounced O_2-evolution in the light (Fig. 2). Since there was no artificial electron acceptor, this proved complete photosynthetic activity of the isolated chloroplasts. With 25 to 35 μmol O_2·mg chlorophyll^{-1}·h^{-1} it was satisfactory according to general ranking. High and repro-

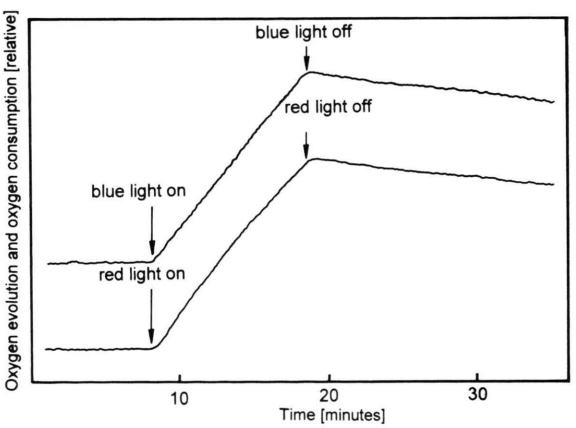

Fig. 2. Oxygen exchange of isolated intact chloroplasts of *Pisum sativum* (95% intact) in darkness and in blue (λ 410–540 nm, T_{max} λ 465 nm) or red (λ 570–700 nm, T_{max} λ 660 nm) light of equal quantum fluence rates of 150 μmol·m^{-2}·s^{-1} determined by polarometry. O_2-evolution in blue light = 24.4 μmol·mg chlorophyll^{-1}·h^{-1}, in red light = 24.7 μmol·mg chlorophyll^{-1}·h^{-1}.

ducible rates of O_2-output in the light were only obtained when glycerate 3-phosphate was applied. $NaHCO_3$ alone yielded sometimes larger, but mostly variable and unstable rates. In darkness following illumination, a small uptake of oxygen could regularly be detected. It was, however, of great variance. It ranged from 5 to about 10% of the oxygen amount released in preceding light.

Mass spectrometric analysis resulted in different responses of $^{16}O_2$ and of $^{18}O_2$, both applied above, and therefore also dissolved in, the aqueous phase. In **initial darkness**, there was no measurable alteration in $^{16}O_2$-concentration, but there was a pronounced continuous decrease in $^{18}O_2$ (Fig. 3).

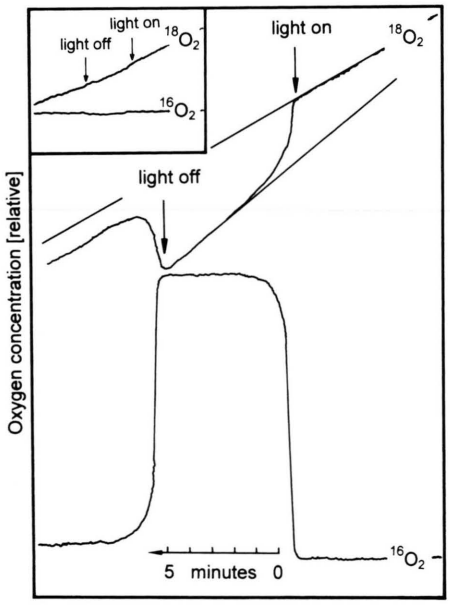

Fig. 3. Oxygen exchange of isolated intact chloroplasts of *Pisum sativum* (93.6% intact) in darkness and in white light of 4 $\mu mol \cdot m^{-2} \cdot s^{-1}$ determined by mass spectrometry. 2 ml chloroplast suspension (density = 45.5 µg chlorophyll/ml = 91 µg chlorophyll/assay) were filled in the reaction chamber and their chloroplasts left settling down for 45 min. They finally formed a monolayer on the teflon membrane at the bottom. The atmosphere in the chamber was air in which part of the natural $^{16}O_2$ was substituted by $^{18}O_2$. The aqueous medium was made up of only $H_2{}^{16}O$-containing buffer. Recording sensitivities were 100 mV for $^{18}O_2$ and 200 mV for $^{16}O_2$. $^{16}O_2$-evolution = approx. 7 $\mu mol \cdot mg\ chlorophyll^{-1} \cdot h^{-1}$, $^{18}O_2$-consumption = approx. 0.8 $\mu mol \cdot mg\ chlorophyll^{-1} \cdot h^{-1}$. Inset: Mass spectrometric determination of $^{16}O_2$ and of $^{18}O_2$ applied to the gas phase over a suspension of heat-denatured (10 min, 100 °C) isolated pea chloroplasts (84 µg chlorophyll/assay). For further details see Materials and Methods. Original recordings.

However, this decrease was measurable also with heat-denatured chloroplasts (Fig. 3, inset). It thus did not depend on chloroplast metabolism, but was due to the technique applied as mentioned above. In the **light**, there was a pronounced liberation of $^{16}O_2$ from water splitting. After about 2 min, the recorded curve levelled off, indicating equilibrium between photosynthetically liberated, metabolically consumed and technically used up $^{16}O_2$. This rate of oxygen exchange dropped sharply after turning the lights off. It took about 2 min, until a new constant trace was reached. This was higher than that before illumination, indicating that the concentration of $^{16}O_2$ in the sample had increased from water oxidation. Illumination also initiated a largely steeper slope of $^{18}O_2$-decrease which turned into a smaller, linear decline within about 2 min. This was still steeper than that in preceding darkness. After light off, the rate of oxygen consumption developed back, not reaching, however, the level of extrapolated decline of $^{18}O_2$ present in darkness before illumination. The faster decline during irradiation and the lower level after the light period clearly indicated extra $^{18}O_2$-consumption of the illuminated preparation. Whether there was also some O_2-uptake in darkness after the light period could not be decided. The method applied did not give unequivocal respective informations.

Reliable quantitative data for **total** oxygen consumption in the light could not be calculated from these recordings, because it had to be expected, that there was simultaneous uptake of $^{16}O_2$. This assumption was supported by the traces of the recordings: Levelling off of $^{18}O_2$-uptake with time coincided with that of $^{16}O_2$-liberation from water oxidation rather closely (see Fig. 3). The former, therefore, might be due to initially increasing, later constant replacement of $^{18}O_2$ from the gas phase by photosynthetically evolved $^{16}O_2$. Although in control experiments, simultaneously applied $^{18}O_2$ and $^{16}O_2$ had been taken up at comparable rates, in present experiments, we had no reliable basis for calculations, since the concentrations of the two isotopes at the site of action were not known: $^{18}O_2$ had to diffuse into the organelle from the gas phase, while $^{16}O_2$ was produced in it. We, therefore, decided, to use as an indicator for oxygen consumption in the light the uptake of $^{18}O_2$, *i.e.* the area, limited by the recorded curve for $^{18}O_2$

and the extrapolation of its slope in preceding darkness, in the following.

Rotenone, antimycin A and cyanide, inhibitors of complexes I, III and IV of the mitochondrial electron transport chain (Singer, 1979), did not prevent uptake of $^{18}O_2$ in the light, neither did SHAM nor propylgallate, inhibitors of the alternative electron pathway in mitochondria (Solomos, 1977; Siedow and Girvin, 1980). Table I shows, that the ratios of "$^{16}O_2$ produced to $^{18}O_2$ consumed" were about 100 to 10 in all cases. We, therefore, considered the observed uptake of $^{18}O_2$ in the light independent of mitochondrial impurities in our preparations. It rather was taken as to indicate oxygen consumption by the intact chloroplasts which – in addition – from these data, did not include oxidases of the regular or the alternative mitochondrial pathways.

Table I. Ratios of "$^{16}O_2$ produced to $^{18}O_2$ consumed" during illumination (λ 448 nm, 16 μmol·m^{-2}·s^{-1}) of isolated intact chloroplasts of *Pisum sativum* exposed to various inhibitors of mitochondrial electron transport chain. Relative data. Mass spectrometric analysis with $^{16}O_2$ in water and gas phase and $^{18}O_2$ in gas phase only.

Inhibitor		^{16}Oxygen produced : ^{18}Oxygen consumed
Control		100 : 10.4 ± 0.6 (n = 16)
Control + 1% Ethanol		100 : 9.6 ± 1.4 (n = 7)
Rotenone	5×10^{-5} M	100 : 9.3
Antimycin A	5×10^{-6} M	100 : 11.3
KCN	10^{-3} M	100 : 9.8
SHAM	10^{-3} M	100 : 9.8
Propylgallate	10^{-3} M	100 : 10.3

The light-dependent $^{18}O_2$-uptake increased with increase in light intensity. This was identical in blue light (λ 448 nm) and in red light (λ 679 nm). At all quantum fluence rates tested, it resembled closely the increase in photosynthetic O_2-evolution at both spectral regions (Fig. 4). The slightly larger increase in $^{18}O_2$-uptake than in $^{16}O_2$-liberation towards the highest fluence rate tested – resulting in a ratio of "$^{16}O_2$ produced to $^{18}O_2$ consumed" of 100 to 10–12 instead of 100 to 8–10 at the lower fluence rates – was not significant.

The light-dependent oxygen consumption was strictly accompanied by electron flow through the photosynthetic machinery. Interruption of this flow by DCMU resulted not only in complete absence of photosynthetic $^{16}O_2$-evolution, but also in complete absence of $^{18}O_2$-consumption. Fig. 5 is an original recording for blue light. Application of red light yielded the same result. DCMU is thought to inhibit electron transfer from bound quinone, Q_B, into the plastoquinone pool. If electrons reducing molecular oxygen originated in the

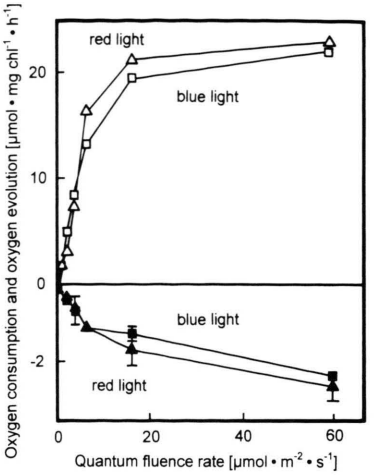

Fig. 4. Dependence on the quantum fluence rate of blue light (λ 448 nm) and of red light (λ 679 nm) of $^{16}O_2$-evolution and of $^{18}O_2$-consumption of isolated intact chloroplasts of *Pisum sativum*. For details of mass spectrometric analysis see Materials and Methods. (Note: μmol O_2·mg chlorophyll^{-1}·h^{-1} = approx. according to calibration [see Materials and Methods]).

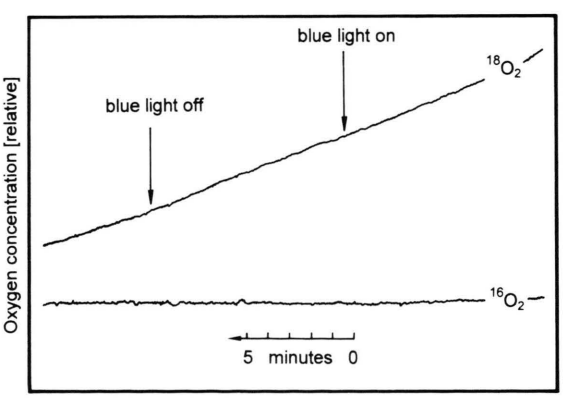

Fig. 5. Alterations in concentrations of $^{16}O_2$ (provided as $H_2{}^{16}O$ and in gas phase) and of $^{18}O_2$ (in gas phase) in suspensions of DCMU-treated (10^{-5} M) isolated intact chloroplasts of *Pisum sativum* (96% intact, 80 μg chlorophyll/assay) in darkness and under blue light (λ 448 nm, 16 μmol·m^{-2}·s^{-1}). Recording sensitivities were 100 mV for $^{18}O_2$ and 200 mV for $^{16}O_2$. For details of mass spectrometric analysis see Materials and Methods. Original recording.

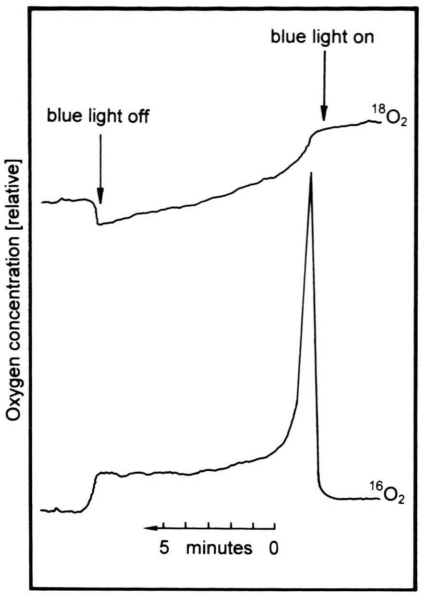

Fig. 6. Alterations in concentrations of $^{16}O_2$ (provided as $H_2{}^{16}O$ and in gas phase) and of $^{18}O_2$ (in gas phase) in suspensions of DBMIB-treated (10^{-5} M) isolated intact chloroplasts of *Pisum sativum* (94% intact, 97 μg chlorophyll/assay) in darkness and under blue light (λ 448 nm, 16 μmol·m^{-2}·s^{-1}). Recording sensitivities were 100 mV for $^{18}O_2$ and 200 mV for $^{16}O_2$. $^{18}O_2$-consumption = approx. 0.9 μmol·mg chlorophyll^{-1}·h^{-1}. For details of mass spectrometric analysis see Materials and Methods. Original recordings.

photosynthetic electron transport chain; they would have had to leave this chain beyond the site of DCMU-action, *i.e.* at or beyond the plastoquinone pool. Of course, the data did not exclude oxygen consumption by reactions dependent on products generated by a complete flow of electrons through the photosynthetic electron transport chain.

This latter assumption was not supported by responses of the isolated chloroplasts to DBMIB. This substance is assumed to affect transfer of electrons from plastoquinone pool to plastocyanine *via* the cytochrome b_6/f-complex (Trebst *et al.*, 1970). Application of 10 μM of this inhibitor to pea chloroplast preparations (Fig. 6) resulted in a high $^{16}O_2$-output immediately after onset of illumination which developed, however, into a very low constant rate within about one minute. Simultaneously, $^{18}O_2$-consumption was increased. As in untreated controls, its decline during irradiation was steeper and the $^{18}O_2$-concentration after illumination was lower than that resulting from extrapolation of the decline in preceding darkness. All responses were identical in blue and in red light. The light-dependent transient of $^{16}O_2$-production could be explained by filling up with electrons from water oxidation of the photosynthetic plastoquinone pool. The subsequent low, but constant rate of $^{16}O_2$-output from little water oxidation might rather be due to passage of electrons to molecular oxygen than to remaining transfer through the cytochrome b_6/f-complex to photosystem I. If so, the data would match involvement of the plastoquinone pool in electron transfer to molecular oxygen as proposed for chlororespiration. The comparatively high uptake of oxygen, resulting in a ratio of "$^{16}O_2$ produced to $^{18}O_2$ consumed" of about 100 to 75, on first view indicated an enhancing effect of DBMIB on oxygen uptake. But this might not be true, because impaired photosynthetic formation of $^{16}O_2$ could lead to consumption of larger portions of $^{18}O_2$ from the gas phase.

As mentioned above, antimycin A, an inhibitor of the cytochrome *bc*-complex of the mitochondrial respiratory electron transport chain (Thierbach and Reichenbach, 1981; Von Jagow and Engel, 1981; Hauska *et al.*, 1983), exhibited no significant influence on $^{16}O_2$-evolution and on $^{18}O_2$-consumption in blue or in red light when applied at 5×10^{-6} M (Fig. 7A = original recording for red light). At the higher concentration of 4×10^{-5} M, it led, however, to pronounced inhibition of photosynthetic $^{16}O_2$-evolution. But even at this high concentration, uptake of $^{18}O_2$, was not comparably impaired (Fig. 7B). This corresponded to reports of Ravenel and Peltier (1991) obtained with *Chlamydomonas reinhardtii.* The result would easiest be explained by influences of antimycin A on the cytochrome b_6/f-complex: Leading to drain off of electrons from plastoquinone pool to oxygen, it would act as proposed in case of DBMIB-treatment, in principle. But such influences do not seem to exist (Wood and Bendall, 1976; Bendall, 1982; Rich, 1984; in context of chlororespiration see *e.g.* Caron *et al.*, 1987; Ting and Owens, 1993 for the diatom, *Phaeodactylum tricornutum*, Mattijs *et al.*, 1984 for the cyanobacterium, *Plectonema boryanum*). Inhibitory influences on photosynthetic gas exchange observed are considered due to effects on cyclic electron flow (Moss and Bendall, 1984).

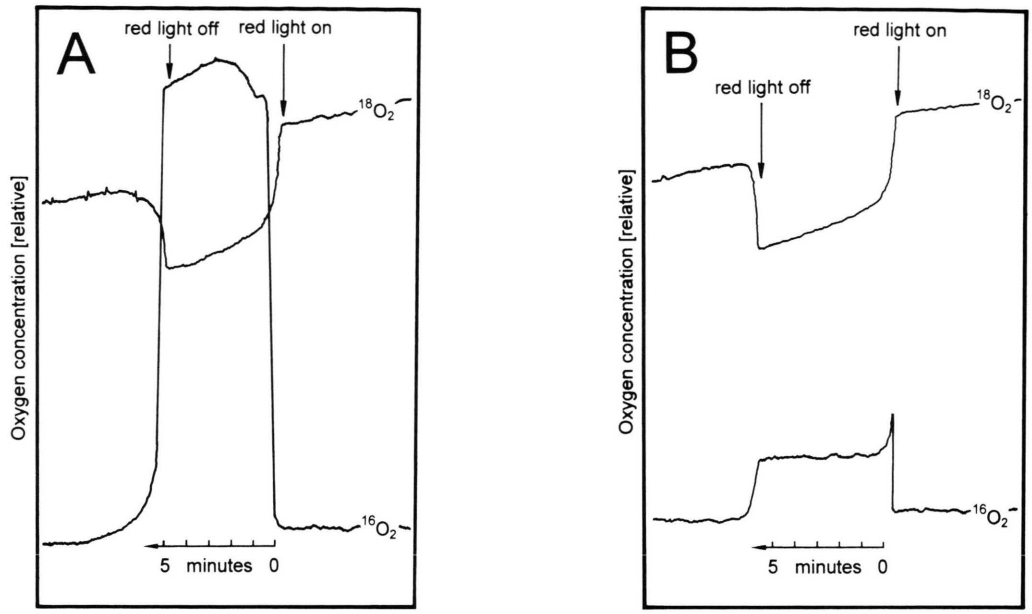

Fig. 7. Influences of antimycin A (A = 5×10^{-6} M ; B = 4×10^{-5} M) on concentrations of $^{16}O_2$ (provided as $H_2{}^{16}O$ and in gas phase) and of $^{18}O_2$ (in gas phase) in suspensions of isolated intact chloroplasts of *Pisum sativum* (96% intact, 46.5 μg chlorophyll/assay) in darkness and under red light (λ 679 nm, 16 μmol·m^{-2}·s^{-1}). Recording sensitivities were 100 mV for $^{18}O_2$ and 200 mV for $^{16}O_2$. $^{16}O_2$-evolution: A = approx. 22 μmol·mg chlorophyll^{-1}·h^{-1}, B = approx. 2.9 μmol·mg chlorophyll^{-1}·h^{-1}, $^{18}O_2$-consumption: A = approx. 2.8 μmol·mg chlorophyll^{-1}·h^{-1}, B = approx. 2.2 μmol·mg chlorophyll^{-1}·h^{-1} For details of mass spectrometric analysis see Materials and Methods. Original recordings.

Final Discussion

Provided our interpretations of the mass spectrometric recordings obtained are correct, and accepting the generally assumed specific effectiveness of the inhibitors applied, isolated intact pea chloroplasts consume molecular oxygen during photosynthesis at non-saturating as well as saturating quantum fluence rates. Oxygen appears to be reduced by electrons originating in light-excited photosystem II and being drained off from plastoquinone pool. This resembles chlororespiration. Photorespiration, another oxygen consuming process in chloroplasts of C_3-plants like pea, is not likely to be involved, decisively. It takes place at low CO_2- and high O_2-concentrations and at high light intensities. In our experiments, O_2-uptake was measured at low light intensities, already, and it increased congruently with photosynthetic O_2-release, resulting in saturation of both reactions at the same quantum fluence rates. Admittedly, this latter statement holds true only when the calculated $^{18}O_2$ is a reliable indication for **total** oxygen consumption at all light intensities tested, *i.e.* when there is no substantially different simultaneous uptake of photosynthetically produced $^{16}O_2$, not recognizable in our experiments. Also O_2-consuming Mehler type reactions, generally discussed as a means to oxidize excess NADPH in chloroplasts, occur only at high light intensities at which production of reducing power exceeds its demand in photosynthesis. Again, this situation does not apply to our experiments. Therefore, in the following, chlororespiration shall be considered to be the O_2-consuming reaction. As outlined comprehensively in Introduction, its pathway is not completely known yet, but inhibition by DCMU and – restricted – also by antimycin A as well as insensitivity to DBMIB have been reported to be characteristic features. Concerning its significance, reoxidation of NADPH in **darkness** has been proposed originally. It was thought to secure proper adjustment of the photosynthetic machinery for a light period to come (Bennoun, 1982; Ravenel and Peltier, 1991; Wilhelm and Duval, 1990; Groom *et al.*, 1993). Our data do not indicate involvement

of NAD(P)H, but there seems to be a **simultaneous** flow of electrons from photosystem II to photosystem I and to molecular oxygen in the light. The constant ratio of "oxygen consumed to oxygen produced" at all quantum fluence rates tested makes it appear an integral part of the photosynthetic light reaction, indeed. At the all together still limited information available, any hypothesis on significance of transfer of light-excited electrons to molecular oxygen will be highly speculative, not least beause we know nothing about the localization of the final oxydase. Giving it a try, we will assume it at the outer side, *i.e.* the stroma side, of the thylakoid membrane. The reaction then would reduce the concentration of oxygen in the vicinity of ribulose 1,5-bisphosphate carboxylase, preventing thereby the enzym's oxygenase activity. This would improve the plant's productivity indirectly. But with this assumption, also a direct influence on photosynthetic efficiency can be constructed: Reduction of oxygen by electrons from plastoquinone pool would increase the pH-gradient across the thylakoid membrane. This would allow enhanced formation of ATP necessary for CO_2-reduction during illumination. Quantitative statements on this cannot be made yet. Respective calculations need information on total oxygen uptake in the light. This is not available. But there are two observations which allow some estimation. First, uptake of the tracer $^{18}O_2$ amounts to about 10% of oxygen liberated by water splitting. Total uptake should be remarkably larger because of the repeatedly mentioned concomitant use of $^{16}O_2$. Second, O_2-uptake is present at all quantum fluence rates tested, – *i.e.* it obviously competes with electron transfer to cytochrome b_6/f-complex and is not just result of an overflow of plastoquinone pool. Therefore, a rather high electron affinity of the pathway leading to molecular oxygen must be expected. Both these data nourish the idea, that a reasonable percentage of light-excited electrons from photosystem II might be transported to molecular oxygen. We certainly feel, that proving or disproving the question wether we will have to distinguish between $NADP^+$-bound and oxygen-bound electron transport in photosynthesis and wether the latter is a regular and vital means to satisfy ATP-demand for CO_2-reduction in photosynthesis is a rewarding task for future research.

With regard to the nature of the oxidase mediating the final electron transfer to molecular oxygen, our data do not extend existing knowledge.

Aside from the main concern of these investigations, a short comment on light-enhanced respiratory gas exchange of several algae shall be added. They respond with a remarkable increase in O_2-uptake specifically to blue light. It is a matter of debate, whether the underlaying reaction resides in the chloroplast or not (for review: Kowallik, 1982). The experiments performed here, were hoped to contribute also to this problem. But their results give no answer. Oxygen uptake of isolated pea chloroplasts was found identical in blue and in red light and, therefore, must be considered a different reaction.

Acknowledgements

The authors thank Prof. Dr. G. H. Schmid for use of mass spectrometer and generous gift of oxygen isotopes, Prof. Dr. K. P. Bader for methodical help and Martina Holt for technical assistence. W. K. is indebted to Deutsche Forschungsgemeinschaft for financial support (Ko 287/10-2).

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