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Dioxygen uptake by isolated thylakoids from lettuce (*Lactuca sativa* L.): simultaneous measurements of dioxygen uptake, pH change of the medium and chlorophyll fluorescence parameters

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Key words: chlorophyll fluorescence, dioxygen uptake, pH changes, thylakoids, water–water cycle

Abstract

The setup has been elaborated for the simultaneous measurements of dioxygen uptake, pH changes, and chlorophyll *a* fluorescence parameters of an isolated thylakoid suspension. Using this equipment we have found at least three kinetically distinguishable components in the response of dioxygen uptake and pH increase to light intensity in the range of 0–1600 μE m^{-2} s^{-1}. The pH changes were not observed in the presence of uncouplers (2 μM valinomycin plus 2 μM nigericin) while O_2 uptake increased by about 10% and F_v/F_m ratio appeared to be unaffected by this treatment. Treatment with DNP-INT, an inhibitor of plastoquinol oxidation, led to a significant reduction of pH increase and O_2 consumption whereas F_v/F_m was impaired only to 71% of the control. Incubation with catalase (580 U/ml) caused a total inhibition of oxygen uptake, while the pH increased and the F_v/F_m ratio decreased to about 60% and 85% of the control, respectively. The addition of catalase after the irradiation period led to an evolution of the same amount of dioxygen as was consumed during the light period. These results show that hydrogen peroxide was formed in the investigated system and accumulated during illumination. On the basis of the obtained data, three sites of dioxygen reduction within isolated thylakoid membranes and the dependence of dioxygen uptake on the photosystem activities were discussed.

Abbreviations: CET – photosynthetic cyclic electron transport around PS 1; chl – chlorophyll; cyt b_6f – cytochrome b_6/f complex; DCMU – 3-(3′,4′-dichlorophenyl)-1,1-dimethyl urea; DCPIP – 2,6-dichlorophenolindophenol; DNP-INT – 2,4-dinitrophenyl ether of iodonitrothymol; F – chlorophyll *a* fluorescence at a particular actinic light intensity; Fd – ferredoxin; FNR – ferredoxin:$NADP^+$ oxidoreductase; LET – photosynthetic linear electron transport; LI – light intensity; PQ – plastoquinone; PQH_2 – plastoquinol; PS – photosystem; ROS – reactive oxygen species; WWC – water–water cycle = pseudocyclic electron flow

Introduction

The photosynthetic electron transport system in chloroplasts of higher plants consists of well-known linear electron transport, with water as a primary electron donor and $NADP^+$ as a terminal electron acceptor, and a cyclic electron transport around Photosystem 1 (PS 1). These two pathways share a sequence of electron carriers such as plastoquinone pool, cytochrome b_6/f complex, plastocyanin, PS 1 complex, and probably ferredoxin (for a review see Bendall and Manasse 1995; Ke 2001). These two pathways operate in cyanobacteria, algae, the bundle-sheath cells of C_4 plants,

and in C_3 plants (Joët et al. 2002 and papers cited therein).

Besides these, at least three other pathways of electron flow in the thylakoids of higher plants have been demonstrated: (i) a cyclic electron transport around PS 2, involving cyt b_{559} and probably PQ pool (Whitmarsh and Pakrasi 1996; Miyake et al. 2002), (ii) a chlororespiratory pathway, where the plastoquinone pool is reduced by a NAD(P)H-dependent dehydrogenase(s) (Burrows et al. 1998; Kofer et al. 1998; Guera et al. 2000; Casano et al. 2001) and can be oxidized by terminal oxidases or peroxidases (Carol et al. 1999; Wu et al. 1999; Casano et al. 2000) or cyt b_{559} (Kruk and Strzałka 1999) and (iii) pseudocyclic electron transport or water–water cycle (Asada 2000). In the last process mentioned the electron carriers are almost the same as in the linear electron transport pathway, except for the terminal step: electrons derived from PS 1 are not involved in the $NADP^+$ reduction but reduce dioxygen to superoxide anion-radical in the so-called Mehler reaction (Arnon 1977; Heber 2002). It is not yet clear where this reaction takes place. In isolated thylakoids, the O_2 reduction is thought to be associated preferentially with the oxidation of the [4Fe–4S] centres in FeS_X or $FeS_{A/B}$ carriers (Asada et al. 1974; Asada 2000) and/or vitamin K_1 (Kruk et al. 2003). In intact chloroplasts, the superoxide formation may also be associated with the oxidation of Fd_{red} or with the activity of FNR (Goetze and Carpentier 1994; Palatnik et al. 1997; Asada 2000; Foyer and Noctor 2000). A potentially harmful superoxide is then detoxified in a sequence of enzymatically-catalyzed reactions, where water molecules are the end-product. The detoxification of superoxide dominates in stroma and/or at the stroma-side of the thylakoid membrane and at least five enzymes (superoxide dismutase, ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase), as well as several scavengers (ascorbate, glutathione, and prenyllipids) are apparently engaged in this process (Asada 2000; Foyer and Noctor 2000; Kruk et al. 2003).

The pseudocyclic electron transport at low and medium light intensities seems to play a key role in tuning the ratio of ATP/NADPH to the rate of CO_2 fixation. Adjustment of the ATP/NADPH ratio is possible also by CET activity, but this can operate predominantly under conditions where electron flow from PS 2 is limited (Asada 2000). This is especially important for C_4 plants, where the optimal ATP/NADPH ratio demanded for CO_2 fixation is higher than could be achieved in LET reactions (Maroco et al. 2000). Makino et al. (2002) suggested that WWC acts as a 'starter' of photosynthesis by a fast generation ΔpH across the thylakoid membrane. On the other hand, Clarke and Johnson (2001) argued that pseudocyclic electron transport prevents an overreduction of electron carries under stress conditions, e.g. at high temperature of environment or under excessive irradiance (Asada 2000; Miyake and Yokota 2000). Both photosystems engaged in this cycle could be de-excited in this way. However, de-excitation leads to the formation of harmful reactive oxygen species ($O_2^{-\cdot}$, H_2O_2) that in intact chloroplasts are removed by endogenous scavenger systems (Asada 2000). The isolated thylakoids may be depleted of many of these scavenging systems and the formed ROS ($O_2^{-\cdot}$, H_2O_2 or $HO_2^{\cdot}$) can oxidise or reduce several integral components of the thylakoids (e.g. Asada et al. 1974), not only at the sites of their production (Herbert et al. 1992; Krieger-Liszkay et al. 2000; Tjus et al. 2001). Moreover, in isolated thylakoids, where energy transfer and/or electron transport systems are impaired, ROS can be produced by pathways other than the Mehler reaction, even at low light intensity. The production of singlet oxygen ($^1O_2$) (Caspi et al. 2000; Zolla and Rinalducci 2002) is a consequence of photosystem overexcitation, while the reaction centre of PS 2 is the site of hydroxyl radical ($HO^{\cdot}$) generation (Navarri-Izzo et al. 1999; Ivanov and Khorobrykh 2003). The same authors presumed that the generation of superoxide and hydrogen peroxide may be also associated with the autoxidation of the PQ pool. As a result of these reactions, dioxygen is consumed which is concealed by dioxygen production by the oxygen evolving system. Net dioxygen uptake may be observed only under low activities of the ROS scavenger systems and/or when electron donors for photosynthetic electron transport, other than water, are present. In this report, we focused on the determination of sites of light-induced dioxygen uptake by isolated lettuce thylakoids and on the determination of a correlation between the rate of dioxygen consumption and the rate of proton uptake from the medium and changes of chlorophyll *a* fluorescence parameters. We have

elaborated the setup which enables the simultaneous monitoring of the rate of dioxygen uptake by isolated thylakoids, changes of pH of the thylakoid suspension and the kinetic parameters of the fluorescence of chlorophyll in the photosynthetic apparatus.

Materials and methods

Isolation of thylakoids

Thylakoids were isolated from lettuce (*Lactuca sativa* L.) leaves, purchased in summer at a local market, by the procedure of Avron (1961) with the modification described by Waloszek and Więckowski (2004).

Setup used in experiments

All measurements were performed using the setup developed for real-time simultaneous measurements of the pH of the medium, the fluorescence of chlorophyll and the dioxygen concentration changes in the aqueous medium of thylakoid suspension, with the possibility to measure also actinic light intensity. The setup was constructed from three commercially available instruments: a high-sensitive pH-meter (Corning 350, USA, with a Cole–Parmer combined microelectrode 55520-08), a Clark-type O_2 electrode (Hansatech CB1D, UK) and a pulse-amplitude-modulated type chlorophyll fluorometer (PAM 101 with 101ED emitter-detector unit, Walz, Effeltrich, Germany). Additionally, silicon photodiode (BPW 43), with a parallel scaling resistor and a ceramic attenuator (transmission of about 0.1% in the range of photosynthetically active radiation) were used for the measurement light intensity ranging from 0.1 to 1600 $\mu E\ m^{-2}\ s^{-1}$. The photodiode voltage signal was scaled in light intensity units by comparison with measurements using phytophotometer (Sonopan, Poland).

The sample was placed in a standard Hansatech Clark-type O_2 electrode vessel DW2. The O_2 electrode junctions and membrane were integrated parts of the vessel, and they were located at the bottom of it, whereas the pH-meter combined electrode was plunged into the same sample. The chlorophyll fluorometer light guide was in optical contact with the sample through one of the apertures in the side of the vessel and the photodiode was in the opposite aperture. This location of the photodiode had some disadvantages, because the intensity of measured light was decreased by absorption by the sample but, on the other hand, it allowed one to measure the intensity in the poorly illuminated part of the sample. This intensity can be critical for some processes, while the light intensity at the front of the sample can be easily measured by another sensor. The sample was thermostated by a water bath with the temperature controlled by ultrathermostat (U2, MLW, Germany), and continuously stirred by an integrated magnetic stirrer. The light-guide illuminator with a halogen incandescent lamp (Schott KL1500 LCD), commonly used as a source of saturating light in Walz chlorophyll fluorometer, with an orange light filter (OG1, Schott, Germany) was the source of actinic light. The elimination of the short-wavelength light was necessary because of the photosensitivity of the AgCl junction of the pH-meter electrode. Additionally, this system allowed for the de- and re-oxygenation of the sample by bubbling with helium or air, respectively.

The data were collected by a 4-channel analogue/digital converter of a data acquisition board (JMBS Development, France), elaborated for chromatographic data collection. This converter covers a large voltage range (−10 to +10 V) with a resolution of 1 μV and practically no drift in time, and makes it possible to change the sampling frequency (1–10 Hz when all four channels are used) and software scaling the digital output signal, so the effective signal can be observed directly in proper units.

The output of the O_2 electrode cannot directly be plugged into the input of the A/D converter because the potentials of the pH-meter electrode and the O_2 electrode were disturbed mutually. The circuit was closed by output signal lines and also by the ground of the power circuit, so one of the apparatus must be electrically separated from the rest of the system. Hansatech O_2 electrode CB1D can be powered by batteries so the electrical separation of the signal of this part of the system was the simplest way to separate the potentials of the pH-meter and O_2 electrode circuits.

The input electric signal was converted into a proportional light intensity signal and this was back converted into the output electric signal by photoresistors in the measuring bridge. For an

input signal in the range of −1 to +1.5 V, the nonlinearity of output signal was not higher than 3% (Figure 1, inset). This signal did not hinder the data collection in one of the channels of the A/D converter. The actinic light source was controlled by the logic outputs of the data acquisition board. The schema of the whole setup is shown in Figure 1.

For data collection and management 'Borwin' (JMBS Development, France), originally described for operation with the A/D converter used, and 'Origin 4.1' (Microcal Software) and self-made software were employed.

Two milliliters of the sample (20 μM chlorophyll) was placed in the measuring vessel and after the addition of all the reagents the initial pH was adjusted to 5.5, using 5 mM HCl or 5 mM NaOH, and then the sample was pretreated with a measuring light of PAM (< 0.5 μE m^{-2} s^{-1}) for at least 5 min.

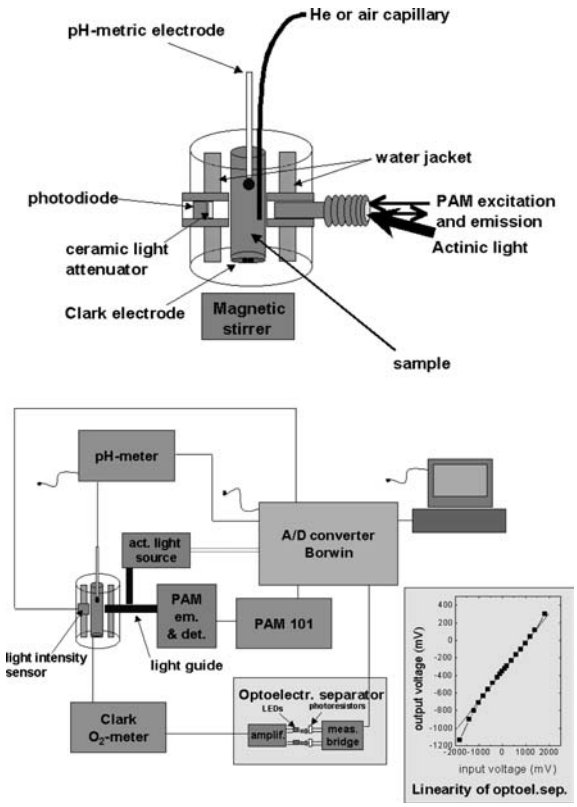

Figure 1. Schema of the experimental setup. A/D converter: data acquisition board Borwin with analogue-to-digital converter and control board; PAM emitter & detector: chlorophyll fluorimeter emitter-detector unit; PAM 101: the basic unit of chlorophyll fluorimeter; LEDs: electroluminescent diodes; amplif.: electronic amplifier driving LEDs; meas. bridge: photoresistor measuring bridge; —— : measuring signal line; – – – –: digital signal guide for actinic light source. Inset: linearity of optoelectronic separator.

Chemicals

The DCMU was from Serva; the valinomycin came from ICN; the nigericin and catalase derived from Sigma; the other chemicals of the analytical grade were purchased from Polskie Odczynniki Chemiczne (Poland); the DNP-INT was a gift from Dr W. Oettmeier (Ruhr University, Bochum, Germany).

Chlorophyll determination

Chlorophyll concentration was determined in 80% acetone as described by Lichtenthaler and Wellburn (1983).

Results

A representative simultaneous record of the three parameters (chlorophyll fluorescence, dioxygen uptake, and pH changes) in the illuminated suspension of dark-adapted isolated thylakoids is shown in Figure 2. As can be expected, illumination caused an increase of F_v during the first few seconds and then the fluorescence intensity declined slightly. The F_v/F_m ratio reached a maximal value of 0.55 during the dark-to-light transition. Under the same conditions, approximately 10.6 μM O_2 was consumed by the thylakoids over 2 min of irradiation with a rate of about 0.27 μmol O_2 min^{-1} μmol chl^{-1}. The addition of the catalase (580 U/ml of sample), after the illumination period, caused an evolution of the dioxygen with amounts slightly higher (32 ± 13%) than was consumed during irradiation. Illumination of dark-adapted thylakoids led also to the de-acidification of the medium; during the initial few seconds the pH increased by about 70 milliunits which corresponded to the proton uptake from the medium at an initial rate of about 3.3 nmol (μmol chl)$^{-1}$ s^{-1}. Turning the light off after 2 min of irradiation led to a decrease, as can be expected, in chlorophyll fluorescence, while the dioxygen uptake was stopped, and the medium acidification started at an initial rate of about 3×10^{-3} pH s^{-1},

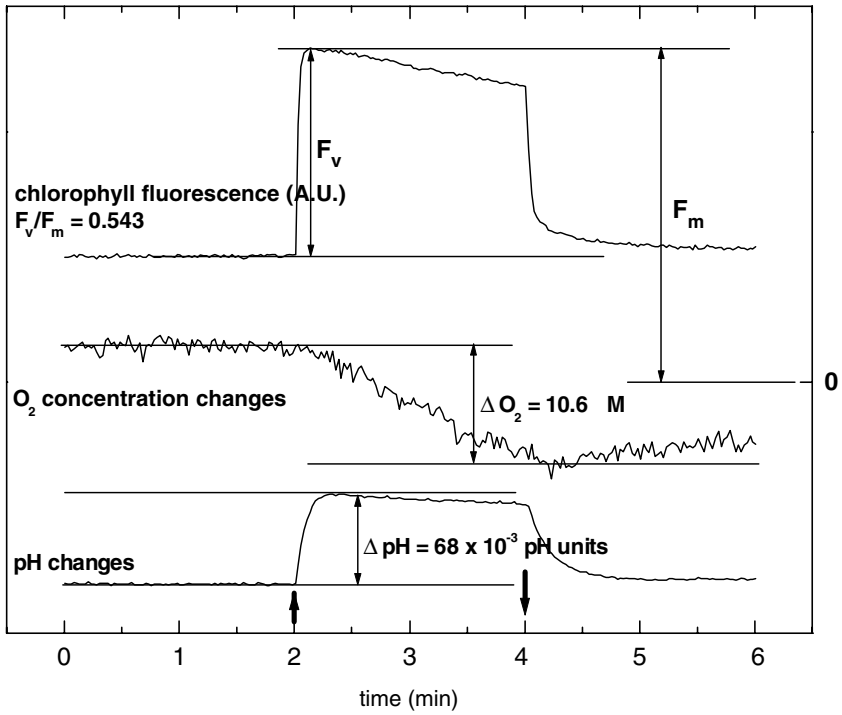

Figure 2. Typical records of light-dependent changes of chlorophyll *a* fluorescence, dioxygen concentration, and the pH of the thylakoid suspension. ↑ - light on, ↓ - light off.

which corresponded to a proton release at the initial rate of about 0.85 nmol (μmol chl)$^{-1}$ s^{-1}. However, when a steady-state level was achieved, the medium become a little less acidic (by about 3×10^{-3} pH) than it was before illumination. The fluorescence changes were comparable in different thylakoid preparations but the extent of O_2 and pH changes varied significantly from preparation to preparation, meaning that these changes depended very much on the quality of isolated thylakoids, although the patterns of changes for different preparations were comparable. The differences in absolute values were registered even if we paid much attention to maintain the same conditions for thylakoid isolation. The reproducibility was not enhanced in thylakoids isolated by the osmotic shock of intact chloroplasts obtained by centrifugation in the Percoll gradient (data not shown). The oxygen uptake was observed only in the suspension of isolated thylakoids since in isolated chloroplasts or leaf fragments, dioxygen releases were observed at a rate of about 0.2 or 0.3 μmol (μmol chl)$^{-1}$ min^{-1} , respectively (data not shown).

Figure 3 illustrates the dependence of the three investigated parameters versus light intensity. The F_v/F_m values increased with the increase in light intensity to about 500 μE m^{-2} s^{-1} and then remained at a constant level, whereas the O_2 consumption and medium deacidification increased with the increase in light intensity to about 1600 μE m^{-2} s^{-1} according to three-phasic kinetics. At the initial phase, the O_2 consumption and medium deacidification increased as the light intensity increased to about 100 and <50 μE m^{-2} s^{-1}, respectively, with the rate constant at about 52 nM O_2 (μE m^{-2} s^{-1})$^{-1}$ and 1.54×10^{-3} pH (μE m^{-2} s^{-1})$^{-1}$, respectively. At the second phase, which took place over a range of light intensity from 100 to 800, or from < 50 to 500 μE m^{-2} s^{-1} for O_2 uptake or medium deacidification, respectively, the rates of these processes were much slower; the rate constants for O_2 uptake and pH increase were calculated to be 7.1 nM O_2 (μE m^{-2} s^{-1})$^{-1}$ and about 0.029×10^{-3} pH (μE m^{-2} s^{-1})$^{-1}$, respectively. At the highest light intensities, > 800 μEm^{-2} s^{-1} for O_2 uptake or > 500 μE m^{-2} s^{-1} for pH increase, the

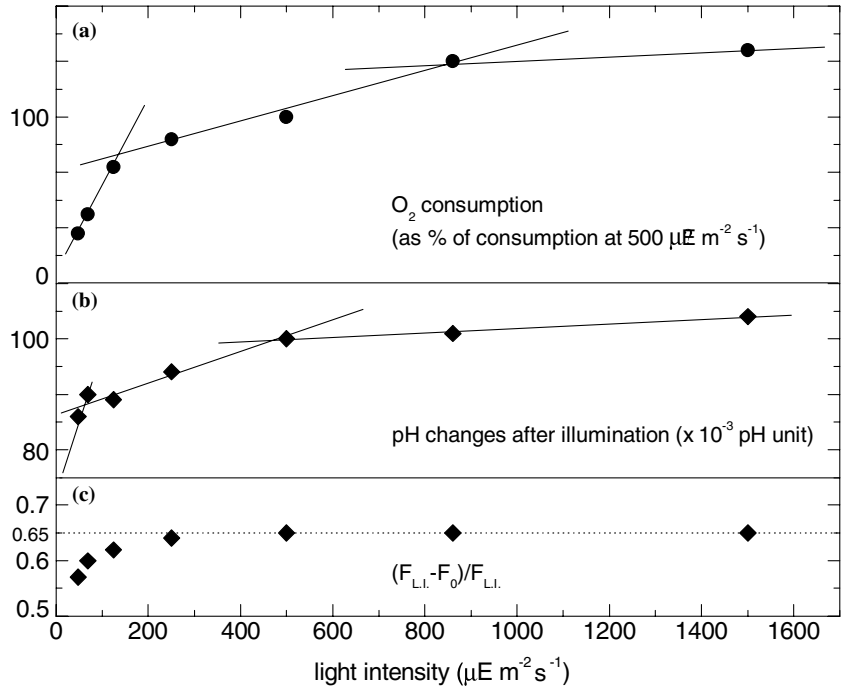

Figure 3. Dioxygen uptake by isolated thylakoids (a) the light-induced pH increase; (b) and changes of F_v/F_m ratio; (c) in response to light intensity. For further details see text.

rates of the investigated processes were calculated to be 1.0 nmol O_2 (nM m^{-2} $s^{-1})^{-1}$ for the O_2 uptake or 6.9×10^{-6} pH (µE m^{-2} $s^{-1})^{-1}$ for the pH increase.

If we assume that the rate constant at the applied lower light intensity is 1, the proportion of the rate constants at higher light intensities will be as 1:0.14:0.02, or 1:0.01:0.004 for the dioxygen uptake or the pH increase, respectively.

In the last set of experiments, we tried to find a mutual correlation between the three processes investigated. As could be expected, the uncouplers (2 µM nigericin + 2 µM valinomycin) caused a total inhibition of the pH changes of the medium, whereas O_2 uptake was enhanced by about 20% and F_v/F_m remained unchanged (Figure 4).

Treatment with 20 µM DNP-INT (2,4-dinitrophenyl ether of iodonitrothymol), an inhibitor of plastoquinol oxidation on the oxidizing site of cyt b_6/f complex (Trebst 1980), led to a significant reduction of pH changes and O_2 uptake (33 and 21% of the controls, respectively), whereas the F_v/F_m ratio was far less impaired (71% of control) (Figure 4).

The addition of the catalase to the thylakoid suspension led to a slight increase in the O_2 concentration in the medium, while the F_v/F_m ratio decreased to about 90%.

Discussion

The elaborated setup enabled a simultaneous determination of the rate of oxygen uptake (or release), chlorophyll fluorescence parameters and pH changes in the non-buffered suspension of isolated thylakoids. This approach should contribute to the determination of the site(s) in the photosynthetic electron transport chain responsible for dioxygen uptake and to the unearthing of the dependence of this process on Photosystem 2 activity and proton gradient generation across the thylakoid membrane. For many decades, it has been general knowledge that chloroplasts or their fragments can utilise molecular oxygen for many light-dependent oxidative processes (see Turner and Britain 1962; Egneus 1975; Malkin and Havaux 2001; and others). Till now, the Mehler reaction has appeared to be the best characterized (Mehler 1951; Mehler and Brown 1952). The oxidation of reduced plastoquinone (Graan and Ort 1984; McCauley and Melis 1986), cytochrome

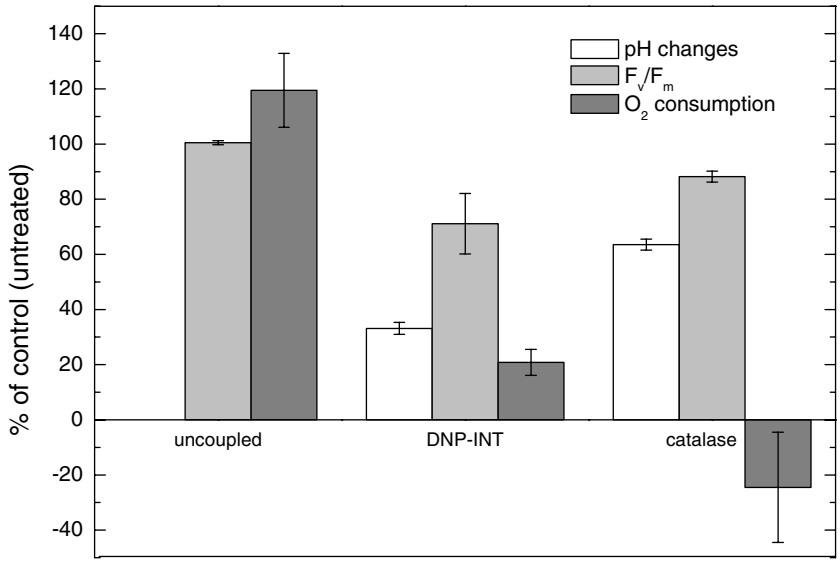

Figure 4. The effects of uncouplers (2 μM valinomycin plus 2 μM nigericin), DNP-INT (20 μM) or the catalase (580 U/ml of sample) on the light-induced pH increase, O_2 consumption and the changes of the F_v/F_m ratio in the suspension of thylakoids. The suspension was illuminated for 2 min by light of 500 μE m^{-2} s^{-1}.

b559LP (Whitmarsh and Pakrasi 1996; Kruk and Strzałka 1999), photosynthetic pigments (Więckowski and Fiedor 1990), some proteins and lipids (Prasad 1996), as well as the transformation of zeaxanthin to violaxanthin (see Demmig-Adams and Adams 1992) under strong light may also be associated with the molecular oxygen uptake. The oxygen uptake associated directly with the photosynthetic electron transport chain is usually masked by concomitant oxygen evolution. Net oxygen consumption is observable only in chloroplasts with at least a partially inactive oxygen-evolving complex and/or deprived of the scavenging systems of reactive oxygen species (see Asada and Takahashi 1987) or under extreme oxidative stress conditions (see Więckowski and Fiedor 1990; Więckowski and Majewska 1990). When the water–water cycle is active in intact chloroplasts, the level of the net dioxygen concentration in the suspension remains constant due to the fast disproportionation of the formed superoxide to water in the Asada cycle (Asada 2000). In the first set of experiments, we showed for the first time that at least three components can be distinguished in the kinetics of light-dependent oxygen uptake in response to light intensity in isolated lettuce thylakoids. We assumed that our thylakoid suspension was deprived of soluble stroma components including those involved in the scavenging of reactive oxygen species. The third component, favoured under excessive irradiance (>900 μE m^{-2} s^{-1}), might be attributed to the oxidation of photosynthetic pigments because pigment photobleaching took place (data not shown). On the basis of the obtained data it is difficult to explain the origin of the two components observed in light intensities that ranged from 0 to 100 μE m^{-2} s^{-1} and from 100 to 900 μE m^{-2} s^{-1}. If we assume that both components occurred also in the suspension irradiated with 500 μE m^{-2} s^{-1} (Figure 4) then they are associated with the formation of superoxide anion radicals and dismutation to H_2O_2 since the net oxygen uptake was not observed in the presence of the catalase, whereas a catalase added after the irradiation period evolved the same amount of dioxygen that was consumed in strong light. Both components were associated with the electron flow from the Photosystem 2 because the net oxygen uptake was undetectable in the presence of DCMU (data not shown) and significantly impaired in the presence of DNP-INT. The preparation was also deprived of Fd and soluble FNR (as was confirmed by electrophoresis, data not shown) and thereby reduced Fd was not involved in the one-electron transfer to O_2. It is generally assumed that the superoxide generation is associated with the activity of Photosystem 1, and

preferentially with the oxidation of Fe–S centres in F_x and $F_{A/B}$ carriers (Takahashi and Asada 1988) and/or with the oxidation of vitamin K_1 (Kruk et al. 2003). We tentatively assume that one component (observable at low light intensity) of the $O_2^{-\cdot}$ formation might be associated with the oxidation of the Fe–S centres, whereas the other (dominant at higher light intensity) might be associated with the $O_2^{-\cdot}$ generation at the step of vitamin K_1. It is presumed that the differences in the rate constants represent the half-time of the reoxidation of these electron carriers; the reoxidation of vitamin K_1 is much slower than that of the F_x centres (Kruk et al. 2003). It might also be associated with differences in the location of these two electron carriers within the thylakoid membrane: Fe–S centres, located near the outer surface of the membrane, are more accessible to O_2, while vitamin K_1 is located deeply within the membrane being less accessible to molecular oxygen. Treatment with DNP-INT, an inhibitor of plastoquinol oxidation at the cyt b_6/f oxidizing site, reduced O_2 uptake and pH change significantly (Figure 4), but not as completely as DCMU treatment (data not shown). It seems that under conditions of PQ pool overreduction, the $O_2^{-\cdot}$ production was associated with the autoxidation of the plastoquinol pool (Ivanov and Khorobrykh 2003; Kruk et al. 2003) and the proton uptake, observed in the medium, was also the effect of proton consumption during the transformation of $O_2^{-\cdot}$ to H_2O_2. The three-phasic kinetics of the medium deacidification did not correspond to the three-phasic kinetics in oxygen consumption since the rate of pH changes versus light intensities preceded the changes in the rate of oxygen uptake. It is well-known that the rate of electron flow through the photosynthetic electron transport chain is regulated by ΔpH across the thylakoid membrane (Slovacek and Hind 1981) but some inertia in the adaptation of the rate electron flow to the ΔpH formation across the thylakoid membrane might occur (Ott et al. 1999). Oxygen uptake at any sites of the thylakoid membrane might be also modified by the presence of scavengers of reactive oxygen species. The rate of oxygen uptake was also not dependent on the generation of *trans*-thylakoid proton gradient. Treatment with uncouplers led even to a slight stimulation of the oxygen uptake. The investigated process was insensitive to antymycin-A (data not shown), so we can assume that in our experimental system, the cyclic electron flow around PS 1 was inactive (due to the absence of ferredoxin) and that the plastoquinone pool was not reduced by NAD(P)H-dehydrogenase systems because the medium was deprived of NAD(P)H. In summary, the obtained data clearly indicated that in isolated and washed thylakoids, at least three principal sites of oxygen uptake occur, among which at least two are associated with the activity of both Photosystem 2- and Photosystem 1-dependent electron flow and with the formation of a superoxide. The oxygen uptake was not associated strictly with the generation of a $[H^+]$ gradient across the thylakoid.

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