Photochemical and Non-Photochemical Quenching of Chlorophyll Fluorescence Induced by Hydrogen Peroxide

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Chlorophyll fluorescence quenching induced by H_2O_2 in intact spinach chloroplasts was investigated with a modulation fluorometer which allows to distinguish between photochemical and non-photochemical quenching components by the so-called saturation pulse method. Residual catalase activity was removed by washing and percoll gradient centrifugation. H_2O_2 was found to induce pronounced photochemical and non-photochemical quenching, characteristic for the action of a Hill reagent, with a half-maximal rate already observed at 5×10^{-6} M. The saturation characteristics and maximal rate of H_2O_2-reduction were very similar to those of methylviologen reduction. H_2O_2-dependent quenching was stimulated by ascorbate and inhibited by cyanide and azide in agreement with previous findings by other researchers that H_2O_2-reduction involves the ascorbate peroxidase scavenging system and that the actual "Hill acceptor" is an oxidation product of ascorbate, *i.e.* monodehydroascorbate or dehydroascorbate. With well-coupled intact chloroplasts reducing CO_2 at 150 μmol (mg Chl)$^{-1}$h^{-1}, iodoacetamide stopped CO_2-dependent O_2-evolution and consequent addition of 10^{-3} M H_2O_2 produced an O_2-evolution rate of 240 μmol (mg Chl)$^{-1}$h^{-1}. It is concluded that light-dependent H_2O_2 reduction is a very efficient reaction in intact chloroplasts. As H_2O_2 formation and consequent reduction also occur *in vivo*, the corresponding quenching should be considered when assimilatory electron flow is estimated from quenching coefficients. It is suggested that proton flux associated with H_2O_2-formation and reduction may be important for the adjustment of appropriate ATP/NADPH ratios required for CO_2-fixation *in vivo*. Furthermore, H_2O_2-reduction may serve as a valve reaction whenever Calvin cycle activity is limited by factors different from NADPH supply, thus protecting against photoinhibitory damage.

Introduction

Chlorophyll fluorescence can give information on almost all aspects of photosynthesis in isolated chloroplasts as well as in intact leaves (for reviews, see ref. [1–4]). Fluorescence yield *in vivo* is lowered by two fundamentally different mechanisms, leading to photochemical and non-photochemical quenching. Photochemical quenching is caused by charge separation at PS II reaction centers, while non-photochemical quenching may be due to a number of other non-radiative de-excitation processes in PS II. Most of non-photochemical quenching is linked to the internal acidification of the thylakoids and is therefore termed "energy-dependent" quenching [5].

Abbreviations: PS, photosystem; DCMU, 3-(3,4-dichlorophenyl-)1,1-dimethylurea; q_P, coefficient of photochemical quenching; q_{NP}, coefficient of non-photochemical quenching.

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Photochemical and non-photochemical quenching components can be determined by the so-called saturation pulse method [6–9], the practical application of which became possible with the introduction of a selective modulation fluorometer [9, 10].

Although photochemical quenching may be considered a reliable measure of PS II charge separation rate, it has been pointed out before that the rate will not necessarily reflect overall assimilatory rate [4]. In previous work, we have considered electron flow to O_2 [4, 11] and cyclic flow around PS II [11–14] to cause "non-assimilatory" photochemical quenching.

O_2-Reduction is an inevitable consequence of aerobic photosynthesis, leading to formation of superoxide, H_2O_2 and other active oxygen species (for reviews, see ref. [15, 16]). The scavenging of active oxygen is of utmost importance for chloroplast protection against oxidative damage. For example, H_2O_2-concentrations as low as 10^{-5} M were found to cause deactivation of Calvin cycle enzymes [17, 18]. Extensive work by Asada and coworkers [16, 19–21] and other researchers [22–24] has established that

chloroplasts not only contain several types of superoxide dismutase [25, 26] but also a very effective H_2O_2-scavenging system, the light-dependent ascorbate peroxidase. Catalase appears to be restricted to the peroxisomes [19, 21, 24, 25].

The present investigation aims at the question to what extent fluorescence quenching is effected by O_2-dependent electron flow, which comprises reduction of O_2 and of the consequently formed H_2O_2. In this first report we will describe the effect of externally added H_2O_2 on fluorescence quenching. It will be shown that the light-driven reduction of H_2O_2 *via* the ascorbate peroxidase is very efficient, matching the reduction of the well known Hill reagent methylviologen. In a following communication [27] the investigation will be extended to the effect of endogenously formed H_2O_2.

Materials and Methods

Intact chloroplasts were isolated from spinach essentially following the method of Jensen and Bassham [28], as modified by Egneus *et al.* [29]. These chloroplasts were further purified by percoll gradient centrifugation, as described by Nakano and Asada [19], to remove residual catalase activity and to further increase the proportion of chloroplasts with intact envelopes. The degree of intactness amounted to 90–95%, as judged by the ferricyanide method [30]. Catalase activity of the final chloroplast preparation was very low, as evidenced by the fact that upon addition of 4 μmol H_2O_2 to a chloroplast suspension containing 150 nmol chlorophyll not more than 10 nmol O_2 was spontaneously formed in the dark. When catalase was added externally, its concentration was 3000 units ml^{-1}. Chloroplasts were suspended isotonically in a reaction medium containing 330 mM sorbitol, 50 mM K-Tricine pH 7.6, 1 mM $MgCl_2$, 0.25 mM Na_2PO_4 and 2 mM $NaHCO_3$. If not stated otherwise, 10 mM Na-ascorbate was present to counteract efflux of intrinsic ascorbate into the medium. In presence of ascorbate the intact chloroplasts displayed high-saturated rates of CO_2-dependent O_2-evolution ranging between 130 and 160 μmol $(mg\ Chl)^{-1}h^{-1}$. Class D chloroplasts were prepared from intact chloroplasts by 30 s exposure to a hypotonic buffer containing 10 mM $MgCl_2$, 10 mM Na-ascorbate and 5 mM K-Tricine pH 7.6, followed by isotonic resuspension by addition of an equal amount of a medium containing the same additions as the medium for intact chloroplasts but at double concentrations. H_2O_2-Solutions of 1 M to 10^{-1} M were freshly prepared every day from a 30% stock solution (Merck). Small aliquots were kept on ice in polypropylene caps each of which was used for one experiment only, to minimize a lowering of effective H_2O_2-concentration by spontaneous decomposition. If not stated otherwise, temperature during measurements was 18 °C and chlorophyll concentration was 60 μg ml^{-1}.

Chlorophyll fluorescence and oxygen evolution were measured as described before [11], with a modulation fluorometer (PAM system, H. Walz, Effeltrich, F.R.G.) equipped with fiber optics and suitable adaptors for a laboratory-built cuvette, containing a side-port Pt/Ag/AgCl electrode. Actinic light was obtained from a tungsten halogen lamp, with a broad red band (about 630–700 nm) selected by RG 630 (Schott) and DT Cyan (Balzers) filters. Repetitive pulses of saturating heat-filtered white light were applied with the help of a relais-controlled fiber illuminator (FL 103, triggered by PAM 103, Walz). Pulse length was 800 ms and pulse intensity 2000 $W \cdot m^{-2}$. The intensity of the modulated measuring beam was very low (about 10^{-2} $W \cdot m^{-2}$) when the *quasi*-dark level of fluorescence yield, F_o, was measured at 1.6 kHz modulation frequency. Simultaneously with the onset of actinic illumination, modulation frequency was automatically switched to 100 kHz by appropriate circuitry in the PAM 102 unit, in order to improve the signal/noise ratio for the kinetic recordings. If not stated otherwise, actinic light intensity was 20 $W \cdot m^{-2}$, as provided at the maximal setting of the light-emitting diode source (102 L) provided with the PAM fluorometer.

Results and Interpretation

In Fig. 1 the basic effect of H_2O_2-induced fluorescence quenching is demonstrated with intact spinach chloroplasts. This example also may serve to illustrate the separation of photochemical and non-photochemical quenching components by the saturation pulse method. When the weak modulated measuring beam is switched on, the *quasi*-dark level fluorescence yield, *Fo*, is monitored. Upon application of a pulse of saturating light the dark-adapted sample displays the maximal fluorescence yield, F_m. F_o and F_m represent extreme values of fluorescence yield, when all PS II reaction centers are open or

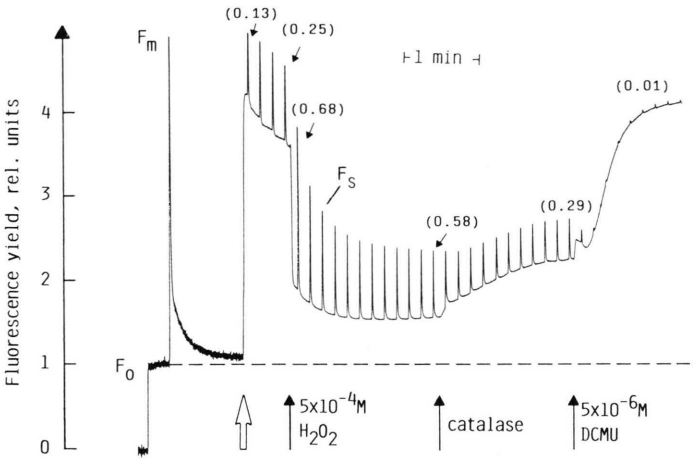

Fig. 1. H_2O_2-induced fluorescence quenching in coupled intact spinach chloroplasts. F_o, dark-level fluorescence yield monitored by extremely weak modulated measuring beam. F_m, maximal fluorescence yield of dark-adapted sample, determined by a pulse of saturating light. Continuous actinic light of 20 $W \cdot m^{-2}$ was turned on at the open arrow. Saturation pulses were applied repetitively every 20 s after onset of continuous illumination. Saturated fluorescence yield, F_s, observed during these pulses is variable, being lowered with respect to F_m by non-photochemical quenching. Photochemical quenching is reflected by the relative amplitude of the spikes produced during a saturation pulse. Some values of the photochemical quenching coefficient, q_P (calculated according to ref. [9]) are written in brackets above the corresponding part of the induction curve.

closed, respectively, without any non-photochemical quenching being induced. When continuous actinic light is switched on, fluorescence yield first rapidly increases and then slowly declines again (Kautsky effect). By repetitive application of saturating light pulses it can be distinguished to what extent the slow fluorescence changes are due to changes in photochemical or in non-photochemical quenching. The lowering of the saturation pulse fluorescence yield, F_s, with respect to F_m is expression of non-photochemical quenching. That part of quenching which can be eliminated by saturation pulses (*i.e.* represented by the "spikes") is due to photochemical quenching (for a detailed definition of the coefficients of photochemical quenching, q_P or q_Q, and of non-photochemical quenching, q_{NP} or q_E, see *e.g.* ref. [4, 8, 9]). When H_2O_2 is added to the stirred chloroplast suspension, there is a large, rapid drop of fluorescence yield followed by a smaller and slower decline. The rapid drop is caused primarily by an increase in photochemical quenching, while the slower decline is paralleled by an increase in non-photochemical quenching and by a small decrease in photochemical quenching. When catalase is added to decompose H_2O_2, photochemical quenching returns almost to its value before H_2O_2 addition while there is only a minor reversion of non-photochemical quenching. Photochemical and most of non-photochemical quenching are effectively eliminated by DCMU, which is known to block electron transfer at the PS II acceptor side.

The rapid increase of fluorescence upon onset of actinic illumination specifically reflects changes in photochemical quenching, as the development of non-photochemical quenching is relatively slow [6–9]. With proper time resolution, this fluorescence increase displays two phases, yielding the so-called O-I-P transient. Fig. 2 shows the effect of H_2O_2, applied at different concentrations to dark-adapted chloroplasts, when the O-I-P transient was induced 5 s following H_2O_2-addition. In Fig. 3 the characteristic fluorescence levels O, I and P are plotted in dependence of the applied H_2O_2-concentration. It is apparent that H_2O_2 affects only the P-level, while O- and I-levels are unaffected. Half-maximal P-level suppression is observed at about 5×10^{-6} M.

The changes in fluorescence characteristics by H_2O_2, as depicted in Fig. 1–3, are typical for the action of Hill reagents, which prevent accumulation of reduced plastoquinone between the two photosystems. According to extensive studies by Asada and co-workers [19–21] and by other researchers [22–24], light-driven H_2O_2-reduction involves the action of the ascorbate peroxidase scavenging system present in chloroplasts. In Fig. 4 the essential role of ascorbate in the H_2O_2-induced quenching reaction is demonstrated for osmotically ruptured chloroplasts, with the intrinsic ascorbate being diluted into the reaction medium. In this experiment the formation of a proton gradient and consequent development of energy-dependent fluorescence quenching was prevented by presence of the protonophore nigericin.

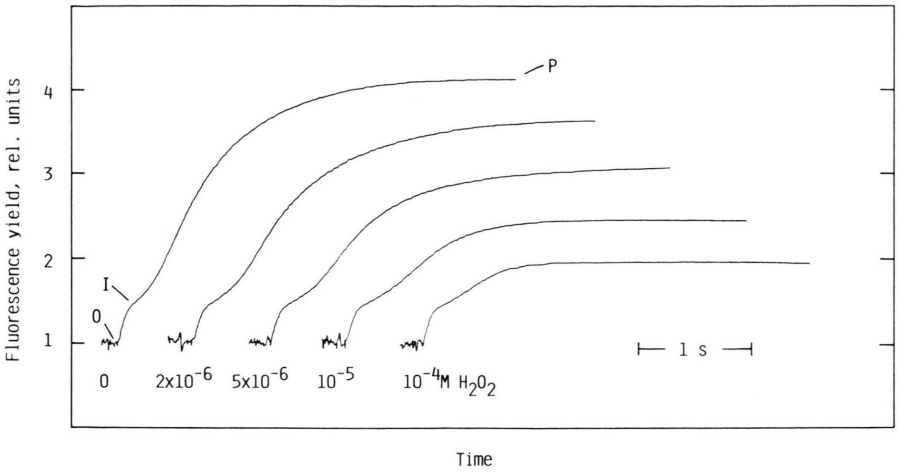

Fig. 2. Rapid rise kinetics of chlorophyll fluorescence in dependence of the H_2O_2-concentration. H_2O_2 was added 5 s before recording of the induction curves. The characteristic fluorescence levels O (dark), I (intermediate) and P (peak) are indicated. Actinic light intensity, 20 $W \cdot m^{-2}$.

Chloroplasts without externally added ascorbate displayed only weak and transient photochemical quenching by H_2O_2 (Fig. 4A). When 5 mM ascorbate was present (during osmotic rupture and the consequent fluorescence experiment) addition of H_2O_2 caused pronounced photochemical quenching which persisted as long as H_2O_2 was available (Fig. 4B).

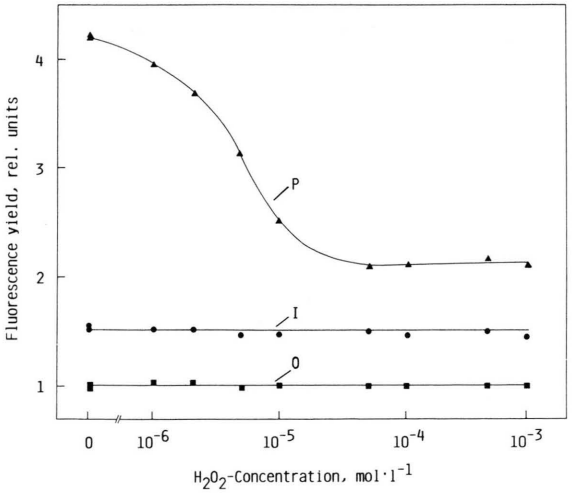

Fig. 3. Dependence of the characteristic fluorescence levels in a rapid induction curve on the H_2O_2-concentration. For conditions, see Fig. 2.

There was an abrupt reversal of quenching when H_2O_2 was consumed. Quenching lasted about two times longer when a twofold amount of H_2O_2 was added. As expected, catalase reversed photochemical quenching by rapid decomposition of the H_2O_2.

H_2O_2-induced fluorescence quenching is suppressed by cyanide and azide, but not by iodoacetamide, in agreement with previous reports showing that cyanide and azide, but not iodoacetamide, inhibit chloroplast peroxidase activity [19–21]. Fig. 5 shows that in presence of 1 mM KCN H_2O_2-induced quenching is almost completely prevented. The same concentration of KCN had no effect on the quenching induced by methylviologen, as shown in Fig. 6. It may be concluded that at the given concentration, the cyanide did not yet affect electron transport at the level of plastocyanine. Hence, the results of Fig. 5 emphasize that H_2O_2 is not directly reduced by the electron transport chain but rather *via* the ascorbate peroxidase system [20, 21]. Accordingly, the actual "Hill acceptor" must be an oxidation product of ascorbate, *i.e.* monodehydroascorbate or dehydroascorbate.

In first approximation, photochemical fluorescence quenching may be considered a relative measure of the rate of charge separation at PS II and, consequently, in the steady state of overall electron flow rate [1–4, 31]. We have compared the photochemical quenching induced by H_2O_2 with that

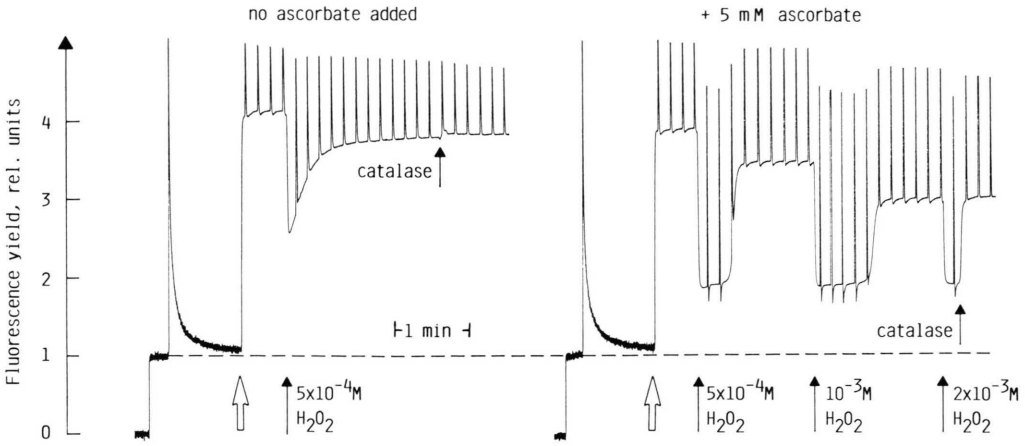

Fig. 4. Stimulation of H_2O_2-induced fluorescence quenching by ascorbate. Class D chloroplasts in the presence of 10^{-7} M nigericin. Chemical additions are indicated at the corresponding arrows.

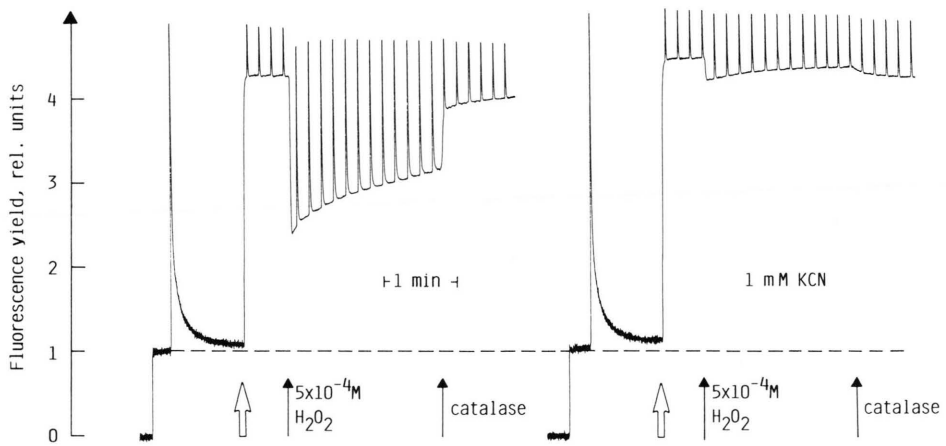

Fig. 5. Inhibition of H_2O_2-dependent fluorescence quenching by cyanide. Intact chloroplasts in presence of 10^{-7} M nigericin. Other conditions as for Fig. 1.

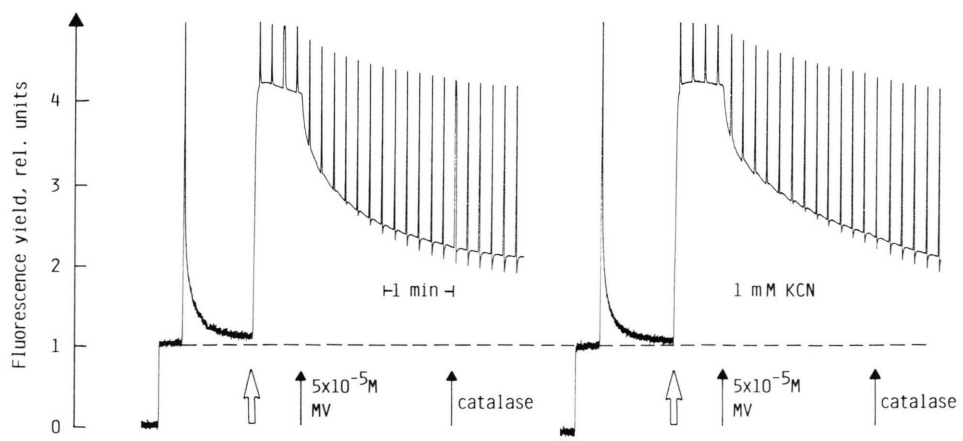

Fig. 6. Methylviologen-induced quenching of chlorophyll fluorescence. Conditions as for Fig. 5. Experiment showing that cyanide at given concentration does not affect methylviologen-dependent electron transport.

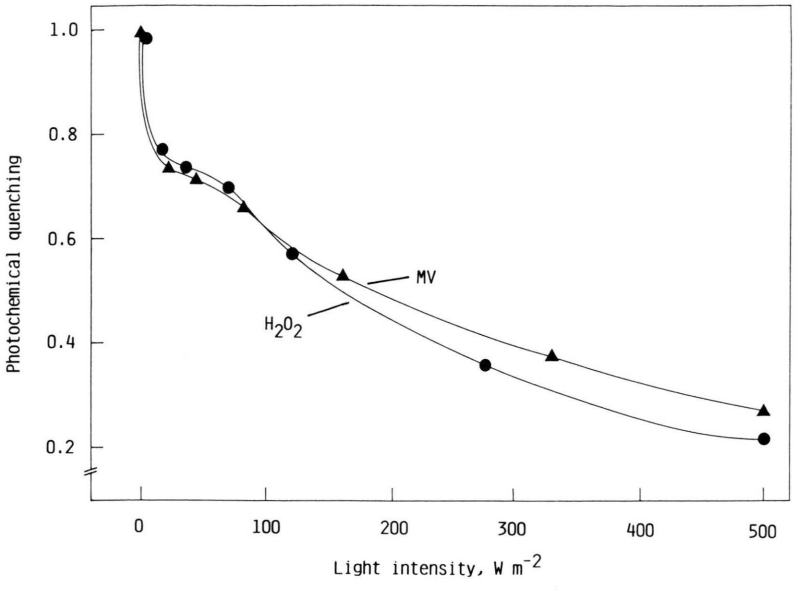

Fig. 7. Light-intensity dependence of the photochemical fluorescence quenching induced by addition of 10^{-3} M H_2O_2 or 10^{-3} M methylviologen to intact, uncoupled spinach chloroplasts. Quenching was determined under the conditions of the experiments in Fig. 5 and 6, except that different intensities of red actinic light were applied (see Materials and Methods). Presence of 10^{-7} M nigericin. In the experiment with methylviologen, 10^{-3} M KCN was added to stabilize the formed H_2O_2.

caused by methylviologen, at a variety of light intensities. To obtain maximal, uncoupled rates, the protonophore nigericin was added. In the methylviologen experiment, 1 mM KCN was added to suppress any residual catalase activity and to inhibit ascorbate peroxidase. The results are shown in Fig. 7. It is apparent that H_2O_2, in combination with the ascorbate peroxidase system, is capable of supporting as high or even somewhat higher rates of electron transport as the well-known and efficient Hill reagent methylviologen. Under the conditions of the experiment of Fig. 7 methylviologen-dependent O_2-uptake saturated at a rate of about 700 μmol O_2 (mg Chl)$^{-1}$h^{-1}. The corresponding light intensity dependence is shown in Fig. 8. These findings confirm previous conclusions by other researchers [19–21, 24], that the potential rate of H_2O_2-scavenging within the chloroplast is very high. As long as this scavenging system is intact, its rate should exceed by far that of any possible formation of H_2O_2.

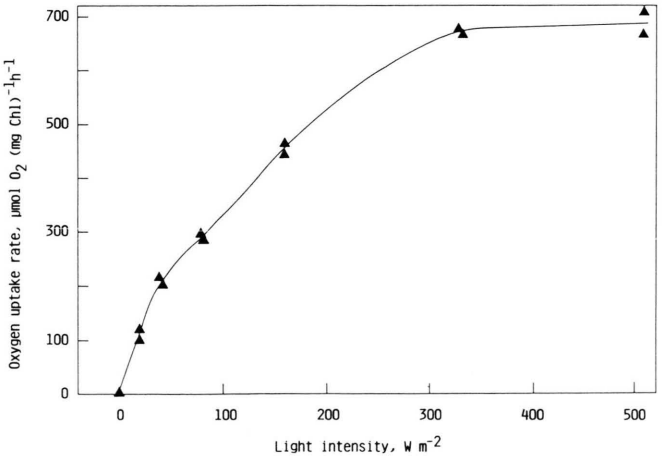

Fig. 8. Light-intensity dependence of oxygen uptake in presence of 10^{-3} M methylviologen, 10^{-7} M nigericin and 10^{-3} M KCN. Conditions as in Fig. 7. Data points from two independent experiments are presented.

Under coupled conditions, a direct comparison of CO_2-dependent O_2-evolution and O_2-evolution linked to reduction of externally added H_2O_2 is possible. Fig. 9 shows simultaneous recordings of O_2-evolution and fluorescence quenching. 20 mM ascorbate was present to provide optimal conditions for the peroxidase. A non-saturating light intensity was chosen to allow an appreciable level of photochemical quenching. The CO_2-dependent rate amounted to 60 μmol O_2 (mg Chl)$^{-1}$h^{-1}, paralleled by q_P = 0.29. When CO_2-fixation was inhibited by iodoacetamide, O_2-evolution was suppressed and q_P decreased to 0.23. This decrease in photochemical quenching is surprisingly small, suggesting that electron flow to non-assimilatory acceptors was almost matching CO_2-dependent flow, once this was inhibited. Actually, it is important to note, that non-photochemical quenching was practically not affected by the inhibition of Calvin cycle. When 10^{-3} M H_2O_2 was added, there was practically no spontaneous O_2-formation, showing that with the given chloroplast preparation residual catalase activity was negligibly small. There was light-dependent O_2-evolution which ceased again, when H_2O_2 was consumed. H_2O_2-dependent rate was 240 μmol O_2 (mg Chl)$^{-1}$h^{-1}, accompanied by q_P = 0.50. Hence, in this particular experiment O_2-evolution linked to reduction of externally added H_2O_2, exceeded CO_2-dependent O_2-evolution (as measured before iodoacetamide addition) by a factor of four. It is remarkable that such a high electron flow rate is possible under coupled conditions when there is no ATP-requirement.

Discussion and Conclusion

Light-dependent reduction of hydrogen peroxide by intact spinach chloroplasts has been previously described by several researchers [19–21, 24]. In particular, the extensive work of Asada and co-workers (for a recent review, see ref. [16]) has emphasized the essential role of the ascorbate peroxidase in the chloroplast stroma for a highly efficient scavenging of H_2O_2. This earlier work was based on measurements of O_2-evolution, O_2-uptake and H_2O_2-consumption by polarography and mass spectroscopy. The main purpose of the present contribution was to investigate in what way chlorophyll fluorescence quenching is influenced by the H_2O_2-scavenging system. Not unexpectedly, it was found that H_2O_2 added to intact chloroplasts causes substantial photochemical and non-photochemical quenching. The properties of this H_2O_2-induced quenching are in ex-

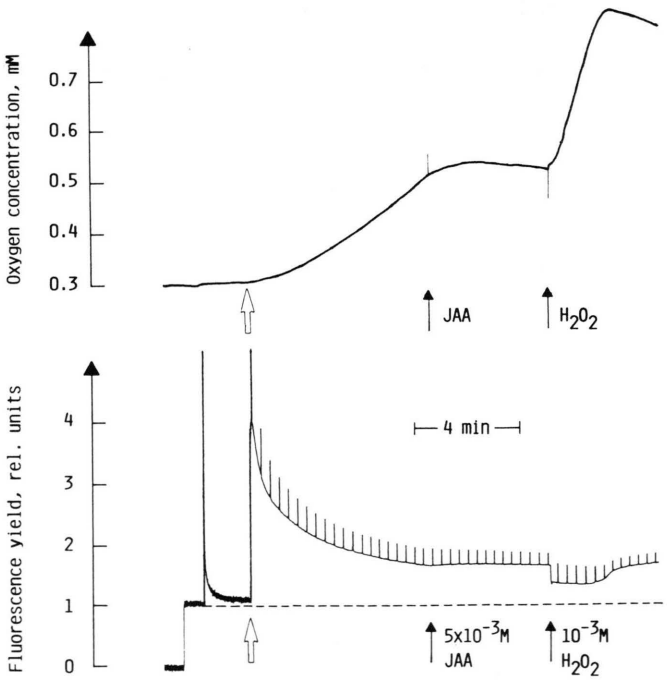

Fig. 9. Simultaneous recordings of modulated chlorophyll fluorescence and polarographically measured oxygen concentration. Effect of iodoacetamide to suppress CO_2-dependent O_2-evolution, and stimulation of O_2-evolution and photochemical quenching by addition of H_2O_2. Actinic light intensity, 150 W·m^{-2}. Temperature, 20 °C. Chlorophyll concentration, 40 μg ml^{-1}.

cellent agreement with the H_2O_2-scavenging mechanism elucidated by previous work:

1) Quenching is stimulated by ascorbate, confirming that ascorbate plays an essential role.
2) Cyanide and azide inhibit quenching, suggesting that H_2O_2 is not directly reduced by the photosynthetic electron transport chain, and that the ascorbate peroxidase is involved.
3) Very low H_2O_2-concentrations are required for stimulation of photochemical quenching. Actually, the concentration for 50% stimulation of 5×10^{-6} M is lower than the previously estimated value of 25×10^{-6} M [24], presumably because the fluorescence response can be more readily measured than O_2-evolution when small amounts of H_2O_2 are rapidly consumed in the light.
4) Substantial photochemical quenching is induced by H_2O_2 even at very high light intensities, showing that the capacity of the H_2O_2-scavenging system is very high. Maximal rates of H_2O_2-reduction match that of methylviologen reduction. Hence, H_2O_2 may be considered a very effective naturally occurring Hill reagent.

These results appear significant for three main reasons:

First, it has been demonstrated that chlorophyll fluorescence, in particular by application of the saturation pulse method, allows insights into the H_2O_2-scavenging mechanism of the chloroplasts. This method can also be used with intact leaves, and in several respects its application may be advantageous to that of polarography and mass spectroscopy. Knowledge about H_2O_2-formation and scavenging is particularly important for an assessment of chloroplast performance under stress conditions when electron consumption in the Calvin cycle is suppressed. H_2O_2-reduction could serve as a valve reaction, preventing photoinhibitory damage of the chloroplasts whenever Calvin cycle is limited by factors different form NADPH. For this function it may be important that H_2O_2-dependent electron flow can display very high rates even under coupled conditions.

Second, the finding of strong quenching induced by small amounts of externally added H_2O_2 raises the question of the possible contribution of such H_2O_2-dependent quenching also in intact leaves under *in vivo* conditions. Univalent reduction of O_2 to superoxide and following dismutation to H_2O_2 and O_2 may be considered an inevitable consequence of photosynthetic electron flow and the efficient scavenging of H_2O_2 is essential for plant survival, as very low H_2O_2-concentrations cause severe inhibition of stroma enzymes [17, 18, 20]. Whenever H_2O_2-formation and scavenging occur in a leaf, this is equivalent to the introduction of a Hill reagent, like methylviologen, with the effect of appreciable fluorescence quenching. Photochemical and non-photochemical quenching produced in this way would overlap quenching related to assimilatory electron flow, and, hence, the determination of this flow would be complicated. In a following communication, we will deal with this aspect in more detail [27].

Third, if H_2O_2-formation indeed is an inevitable consequence of aerobic photosynthetic electron transport, then this is also true for the vectorial proton flux linked to formation and scavenging of H_2O_2. The resulting proton motive force will contribute to ATP-synthesis, and this contribution may be important for the establishment of an overall ATP/$2e^-$ ratio of 1.5 required for CO_2-fixation in C3-plants. Linear electron flow to NADP is believed to provide an ATP/$2e^-$ of 1.33 and it is a matter of controversy whether additional ATP-formation is primarily due to pseudocyclic or cyclic phosphorylation [29, 32, 33] or linked to a Q-cycle involving the Cyt *b/f* complex [34]. Whether H_2O_2-reduction carries sufficient flux *in vivo* to be of any relevance for ATP-synthesis can not be decided on the basis of the above data. This question will be addressed in a following publication [27].

Acknowledgements

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