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

Christian Neubauer and Ulrich Schreiber
Lehrstuhl Botanik I, Universität Würzburg, Mittlerer Dallenbergweg 64, D-8700 Würzburg, Bundesrepublik Deutschland
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Chlorophyll fluorescence quenching induced by H$_2$O$_2$ 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$_2$O$_2$ 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 $5 \times 10^{-7}$ M. The saturation characteristics and maximal rate of H$_2$O$_2$-reduction were very similar to those of methylviologen reduction. H$_2$O$_2$-dependent quenching was stimulated by ascorbate and inhibited by cyanide and azide in agreement with previous findings by other researchers that H$_2$O$_2$-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 $\mu$mol (mg CHl)$^{-1}$h$^{-1}$, iodoacetamide stopped CO$_2$-dependent O$_2$-evolution and consequent addition of $10^{-5}$ M H$_2$O$_2$ produced an O$_2$-evolution rate of 240 $\mu$mol (mg CHl)$^{-1}$h$^{-1}$. It is concluded that light-dependent H$_2$O$_2$ reduction is a very efficient reaction in intact chloroplasts. As H$_2$O$_2$ 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$_2$O$_2$-formation and reduction may be important for the adjustment of appropriate ATP/NADPH ratios required for CO$_2$-fixation in vivo. Furthermore, H$_2$O$_2$-reduction may serve as a valve reaction whenever Calvin cycle activity is limited by factors different from NADPH supply, thus protecting against photo-inhibitory 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.

Reprint requests to Dr. U. Schreiber.
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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$_2$O$_2$ 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$_2$O$_2$-concentrations as low as $10^{-7}$ 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$_2$O$_2$-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$_2$O$_2$. In this first report we will describe the effect of externally added H$_2$O$_2$ on fluorescence quenching. It will be shown that the light-driven reduction of H$_2$O$_2$ 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$_2$O$_2$.

### 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$_2$O$_2$ 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$_3$PO$_4$ and 2 mM NaHCO$_3$. If not stated otherwise, 10 mM Na-ascorbate was present to counteract efflux of intrinsically 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$_2$O$_2$-Solutions of 1 mM to 10$^{-1}$ mM 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$_2$O$_2$-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·m$^{-2}$. The intensity of the modulated measuring beam was very low (about 10$^{-2}$ W·m$^{-2}$) when the quasi-dark level of fluorescence yield, F$_0$, 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·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$_2$O$_2$-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, F$_0$, is monitored. Upon application of a pulse of saturating light the dark-adapted sample displays the maximal fluorescence yield, F$_m$. F$_0$ and F$_m$ represent extreme values of fluorescence yield, when all PS II reaction centers are open or
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 \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \), photochemical quenching returns almost to its value before \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \), applied at different concentrations to dark-adapted chloroplasts, when the O-I-P transient was induced 5 s following \( \text{H}_2\text{O}_2 \)-addition. In Fig. 3 the characteristic fluorescence levels O, I and P are plotted in dependence of the applied \( \text{H}_2\text{O}_2 \)-concentration. It is apparent that \( \text{H}_2\text{O}_2 \) affects only the P-level, while O- and I-levels are unaffected. Half-maximal P-level suppression is observed at about \( 5 \times 10^{-7} \text{ M} \).

The changes in fluorescence characteristics by \( \text{H}_2\text{O}_2 \), 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 \( \text{H}_2\text{O}_2 \)-reduction involves the action of the ascorbate peroxidase scavenging system present in chloroplasts. In Fig. 4 the essential role of ascorbate in the \( \text{H}_2\text{O}_2 \)-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.
Chloroplasts without externally added ascorbate displayed only weak and transient photochemical quenching by H$_2$O$_2$ (Fig. 4A). When 5 mM ascorbate was present (during osmotic rupture and the consequent fluorescence experiment) addition of H$_2$O$_2$ caused pronounced photochemical quenching which persisted as long as H$_2$O$_2$ was available (Fig. 4B).

There was an abrupt reversal of quenching when H$_2$O$_2$ was consumed. Quenching lasted about two times longer when a twofold amount of H$_2$O$_2$ was added. As expected, catalase reversed photochemical quenching by rapid decomposition of the H$_2$O$_2$.

H$_2$O$_2$-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$_2$O$_2$-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$_2$O$_2$ 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$_2$O$_2$ with that
Fig. 4. Stimulation of H$_2$O$_2$-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$_2$O$_2$-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.
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 $\text{H}_2\text{O}_2$, 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 $\text{O}_2$-uptake saturated at a rate of about 700 $\mu$mol $\text{O}_2$ (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 $\text{H}_2\text{O}_2$-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 $\text{H}_2\text{O}_2$. 

Fig. 7. Light-intensity dependence of the photochemical fluorescence quenching induced by addition of $10^{-3}$ M $\text{H}_2\text{O}_2$ 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 $\text{H}_2\text{O}_2$.

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 CO₂-dependent O₂-evolution and O₂-evolution linked to reduction of externally added H₂O₂ is possible. Fig. 9 shows simultaneous recordings of O₂-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 CO₂-dependent rate amounted to 60 μmol O₂ (mg Chl)⁻¹ h⁻¹, paralleled by qP = 0.29. When CO₂-fixation was inhibited by iodoacetamide, O₂-evolution was suppressed and qP decreased to 0.23. This decrease in photochemical quenching is surprisingly small, suggesting that electron flow to non-assimilatory acceptors was almost matching CO₂-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 H₂O₂ was added, there was practically no spontaneous O₂-formation, showing that with the given chloroplast preparation residual catalase activity was negligibly small. There was light-dependent O₂-evolution which ceased again, when H₂O₂ was consumed. H₂O₂-dependent rate was 240 μmol O₂ (mg Chl)⁻¹ h⁻¹, accompanied by qP = 0.50. Hence, in this particular experiment O₂-evolution linked to reduction of externally added H₂O₂, exceeded CO₂-dependent O₂-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 H₂O₂. This earlier work was based on measurements of O₂-evolution, O₂-uptake and H₂O₂-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 H₂O₂-scavenging system. Not unexpectedly, it was found that H₂O₂ added to intact chloroplasts causes substantial photochemical and non-photochemical quenching. The properties of this H₂O₂-induced quenching are in ex-

![Fig. 9. Simultaneous recordings of modulated chlorophyll fluorescence and polarographically measured oxygen concentration. Effect of iodoacetamide to suppress CO₂-dependent O₂-evolution, and stimulation of O₂-evolution and photochemical quenching by addition of H₂O₂. Actinic light intensity, 150 W·m⁻². Temperature, 20 °C. Chlorophyll concentration, 40 μg ml⁻¹.](image-url)
excellent agreement with the H$_2$O$_2$-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$_2$O$_2$ is not directly reduced by the photosynthetic electron transport chain, and that the ascorbate peroxidase is involved.
3) Very low H$_2$O$_2$-concentrations are required for stimulation of photochemical quenching. Actually, the concentration for 50% stimulation of $5 \times 10^{-6}$ M is lower than the previously estimated value of $25 \times 10^{-6}$ M [24], presumably because the fluorescence response can be more readily measured than O$_2$-evolution when small amounts of H$_2$O$_2$ are rapidly consumed in the light.
4) Substantial photochemical quenching is induced by H$_2$O$_2$ even at very high light intensities, showing that the capacity of the H$_2$O$_2$-scavenging system is very high. Maximal rates of H$_2$O$_2$-reduction match that of methylviologen reduction. Hence, H$_2$O$_2$ 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$_2$O$_2$-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$_2$O$_2$-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$_2$O$_2$-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$_2$O$_2$-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$_2$O$_2$ raises the question of the possible contribution of such H$_2$O$_2$-dependent quenching also in intact leaves under in vivo conditions. Univalent reduction of O$_2$ to superoxide and following dismutation to H$_2$O$_2$ and O$_2$ may be considered an inevitable consequence of photosynthetic electron flow and the efficient scavenging of H$_2$O$_2$ is essential for plant survival, as very low H$_2$O$_2$-concentrations cause severe inhibition of stroma enzymes [17, 18, 20]. Whenever H$_2$O$_2$-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$_2$O$_2$-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$_2$O$_2$. 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 C$_3$-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$_2$O$_2$-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].

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