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Variable thermal emission and chlorophyll fluorescence in photosystem II particles

(photoacoustic spectroscopy/variable fluorescence/plastoquinone/Mn/photosynthesis)

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ABSTRACT **In photosynthetic systems, the absorbed light energy is used to generate electron transport or it is lost in the form of fluorescence and thermal emission. While fluorescence can be readily measured, the detection of thermal deactivation processes can be achieved by the photoacoustic technique. In that case, the pressure wave generated by the thermal deactivations in a sample irradiated with modulated light is detected by a sensitive microphone. The relationships between the yield of fluorescence and thermal emissions measured simultaneously were analyzed by using a spinach photosystem II (PSII)-enriched preparation. It is shown that the quenching of fluorescence due to the photochemical activity of the preparations (photochemical quenching) increases in proportion to the fraction of thermal deactivations that is not immediately released as heat but is stored in photochemical intermediates (energy-storage yield) as the intensity of the photoacoustic modulated measuring beam (35 Hz) is decreased. Maximal levels of fluorescence and thermal emissions were both decreased in similar proportions upon photoreduction of pheophytin (Pheo), the primary acceptor of PSII. The variable components of fluorescence and thermal emissions were strongly decreased upon depletion of Mn from the Mn complex that catalyzes water oxidation and were recovered proportionally during reconstitution with Mn^{2+} at various Mn^{2+}/reaction center ratios. Finally, depletion of Mn from the Mn complex together with the Fe of the Q_A–Fe–Q_B complex that is composed of the secondary quinone acceptors of PSII resulted in an increased initial level of fluorescence F_o and in the loss of the variable components of fluorescence and thermal emissions. The initial F_o and the variable components could be partially recovered by reconstitution of both donor and acceptor sides with Mn^{2+}, Co^{2+}, HCO_3^- and plastoquinone. It is concluded that the photochemical fluorescence quenching is correlated with a simultaneous "quenching" of a variable component of thermal emission. It is proposed that the measured component of variable thermal emission is related to the decay of the pair [$P680^+$ $Pheo^-$]. The suggestion is also made that a bicarbonate-induced protonation of reduced Q_A or Q_B or conformational change in the PSII complex, or both, adds an additional entropic factor to the variable thermal emission component.**

In the photosynthetic apparatus, the absorbed light energy is either used to generate electron transport that is initiated by charge separation at the level of photosystems I and II (PSI and PSII), or the energy is lost through fluorescence and thermal emission. Fluorescence can be readily measured, but photoacoustic (PA) spectroscopy has been advantageously used to study the thermal deactivation processes in photosynthetic materials (1). In that case, a solid or semisolid sample is irradiated with modulated light in a closed cell. The thermal deactivations generate a periodic heat flow in the surrounding gas medium that produces a pressure wave transduced into an electric signal by a sensitive microphone. Most usefully, the difference between the thermal emission yields of active and inactive samples allows the determination of the photochemical energy-storage yield (2). In most studies, the inactive sample with closed reaction centers was obtained by superimposition of a nonmodulated background light of saturating intensity on the low-intensity modulated measuring beam (3–6). The energy-storage yield has been correlated with the electron transport status (7, 8), and it was suggested that electron transfer from water to the plastoquinone (PQ) pool was responsible for the stored energy (8–10).

Similarly, part of the fluorescence emission (the so-called variable fluorescence) is closely related to the redox state of the secondary acceptors Q_A and Q_B in PSII and therefore is greatly influenced by the electron transport status (11). Thus, a strong interdependence between the yield of energy storage and the yield of variable fluorescence can be suggested (10). The correlation between chlorophyll (Chl) fluorescence and the PA signal has been studied in intact leaves (12, 13). However, the interpretations were complicated by the participation of PSI in the thermal signal and by a photobaric contribution due to oxygen evolution.

A PSII submembrane fraction depleted of PSI should constitute the simplest model to study the relationship between variable fluorescence emission and thermal energy storage. The thermal emission behavior of such preparations has been studied in detail by this laboratory (8, 14). In this report, we provide a clear demonstration that the fluorescence quenching due to the photochemical activity of PSII (photochemical quenching) parallels an equivalent "quenching" of a variable component of thermal emission. Further, both the variable fluorescence emission and the variable thermal emission were controlled by the redox state of the acceptor and donor sides of the PS. It is also suggested that a bicarbonate-induced protonation of reduced Q_A and Q_B or conformational change in the PSII complex, or both, adds an entropic factor to the variable thermal emission that is not detected in the fluorescence counterpart.

MATERIALS AND METHODS

Chloroplasts were isolated from spinach leaves as described (15). Isolation of the "heavy" oxygen-evolving subchloroplast particles enriched in PSII, designated DT-20, was carried out as described (16) by centrifugation at 20,000 × *g* of a chloroplast suspension treated with 0.4% digitonin and 0.15% Triton X-100. Chl concentration was determined as described (17), and the particles were found to evolve O_2 at

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Abbreviations: PS, photosystem; Chl, chlorophyll; PA, photoacoustic; Pheo, pheophytin; PQ, plastoquinone.

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a rate of 250–300 μmol per mg of Chl per hr under saturating light in the presence of 0.3 mM $K_3Fe(CN)_6$ and 0.2 mM phenylene-*p*-benzoquinone. The concentration of PSII reaction centers was calculated as described (18–21) from the value of photoinduced absorbance changes at 685 nm (ΔA_{685}) related to photoreduction of pheophytin (Pheo), the primary acceptor of PSII (16, 18, 20), and from that at ΔA_{678} related to photooxidation of the primary electron donor P680 (21). The DT-20 samples contained 80–100 Chl molecules per one P680 or one photoreducible Pheo and contained 15,500–16,000 Chl molecules per one P700.

The complete (>95%) extraction of Mn from DT-20 was reached as described (19). DT-20 particles at 50 μg of Chl per ml were incubated for 1 hr at 2°C in the medium containing 1 M Tris·HCl (pH 8.0) and 0.5 M $MgCl_2$. Then the DT-20 particles were precipitated at 20,000 × *g*. The pellet was washed twice by resuspending to 10 μg of Chl per ml and centrifugation, first in 0.8 M Tris·HCl, pH 8.0/10 μM EDTA and then in 20 mM Tris·HCl, pH 8.0/35 mM NaCl.

Simultaneous extraction of Fe and Mn was carried out by incubating PSII preparations (Chl at 50 μg/ml) in 20 mM Tris·HCl, pH 8.0/1 M $LiClO_4$ with 3 mM *o*-phenanthroline for 3 hr at 2°C, followed by centrifugation (20,000 × *g* for 40 min) and dialysis (18 hr at 2°C) of the pellet against 20 mM Tris·HCl, pH 8.0/35 mM NaCl/20 μM EDTA (20).

In the reconstitution experiments with Mn or with Mn and Co, the Fe- and Mn-depleted PSII particles (Chl at 100 μg/ml) in 20 mM Tris·HCl, pH 8.0/0.025% sodium cholate/0.1 mM EDTA/2 mM HCO_3^- were incubated for 24 hr at 2°C in an anaerobic solution containing 0.1 mM $MnCl_2$ or 0.05 mM $MnCl_2$ and 0.1 mM $CoCl_2$ with or without 0.3 mM PQ (PQ-9) and then were dialyzed for 2 days against 20 mM Tris·HCl, pH 8.0/0.025% sodium cholate/1 mM EDTA (20, 22).

For simultaneous fluorescence and PA measurements, either DT-20 particles were used as a suspension in the PA cell at a concentration of 200 μg of Chl per ml in 120 μl of 20 mM Tris·HCl, pH 7.8/35 mM NaCl/2 mM MgCl, or 200 μl of the various preparations (concentration of 200 μg of Chl per ml) was aspirated on a nitrocellulose filter (Millipore, AA type, 0.4 μm pore size) using a gentle vacuum. The filter was cut to the proper dimensions for introduction into the PA cell. Measurements were made with a laboratory-constructed instrument composed of a MTEC PA cell connected to an EG & G/Princeton Applied Research model 5210 lock-in amplifier. The PA measuring beam was provided by a 150-W Xenon lamp (ILC Technology, Sunnydale, CA). The wavelength was selected by a monochromator [Photon Technology International (Princeton, NJ), model PT1 01-001 SF] at 680 nm, and the beam was modulated to 34 Hz with a mechanical chopper (Boston Electronics, Brookline, MA) and introduced into one arm of a quadrifurcated fiber-optic guide. The light intensity of this modulated beam was controlled with neutral density filters. Two arms of the fiber-optic guide were connected to a PAM-101 chlorophyll fluorometer (Walz, Effeltrich, FRG) for simultaneous measurements of fluorescence. The remaining arm was used to provide the nonmodulated background illumination of saturating intensity from the Walz KL1500 illuminator.

RESULTS AND DISCUSSION

Typical traces corresponding to the simultaneous measurement of fluorescence and thermal emission in a PSII preparation are presented in Fig. 1 (aerobic conditions). The 1.6-kHz excitation beam from the Walz fluorometer is used to induce a fluorescence rise to the initial level F_i. This level provides a good approximation of F_o. However, the PA measuring excitation beam is too weak to generate a detectable PA signal. The latter is obtained by subsequent addition of a 680-nm modulated light (35 Hz, 5 W·m^{-2}), which produces a control level of PA signal (Q_c) and a variable level of fluorescence (F_v). The closure of part of the PSII reaction centers by the PA measuring beam is demonstrated by the occurrence of F_v. Total closure of the reaction centers is obtained with the further addition of a white nonmodulated saturating beam that leads to the maximal levels of modulated fluorescence (F_m) and thermal emission (Q_m).

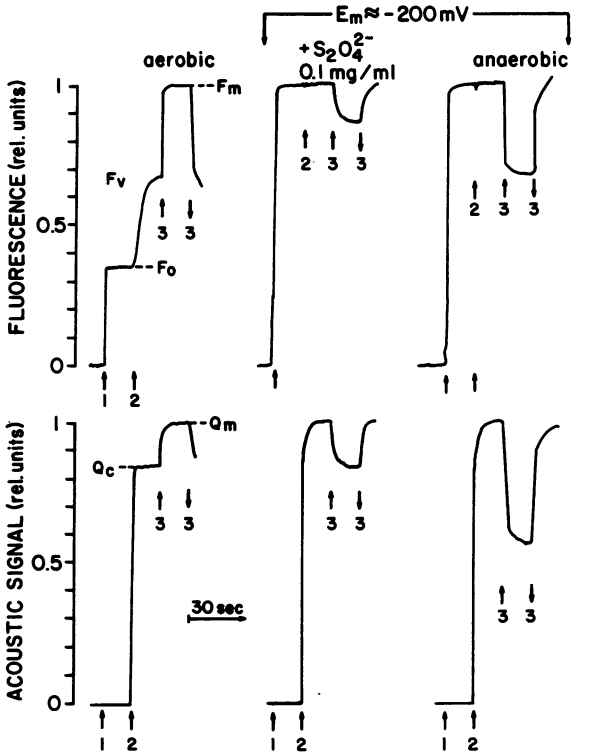

FIG. 1. Typical traces for simultaneous measurement of fluorescence and thermal emissions in the PSII particles. Up and down arrows indicate light on and off, respectively. Numbers under the arrows: 1, fluorescence probe beam; 2, PA measuring beam (680 nm, 3 W·m^{-2}, 34 Hz); 3, saturating background illumination W·m^{-2}. PSII particles were in suspension at a Chl concentration of 200 μg·ml^{-1}. Similar data were obtained with the preparation aspirated on a nitrocellulose filter. Aerobic conditions were obtained with the untreated native preparations, reducing conditions were obtained with addition of 0.1 mg of $S_2O_4^{2+}$ per ml, and anaerobic conditions were obtained with the addition of 10 mM glucose, 50 units of glucose oxidase per ml, and 1000 units of catalase per ml. rel., Relative. $Q_c = Q_v$ (see text).

The relation between fluorescence and PA levels can be characterized if a photochemical quenching parameter, $q_p = [(F_m - F_v)/F_m] \times 100\%$, is used in conjunction with the equivalent PA parameter—namely, $[(Q_m - Q_c)/Q_m] \times 100\%$. This PA parameter is the term used to quantify the photochemical energy storage (ϕ_f') (12). From the above considerations, it can be assumed that the level Q_c corresponds to a variable level of modulated thermal emission and that Q_c should rather be identified as Q_v in relation to the fluorescence level F_v. It has been shown on several occasions that ϕ_f' strongly depends on the PA measuring beam intensity because of the gradual closure of PSII reaction centers and of the concurrent increase of Q_v as the light intensity is raised (3, 8, 9, 14). This phenomenon is shown in Fig. 2*A* where the reciprocal of ϕ_f' is also plotted against the PA measuring beam intensity. A value of ϕ_f' with all of the reaction centers in the open state (ϕ_{fo}') can be obtained from the value of the semireciprocal plot at the ordinate (8, 9, 14). In the present case, this value is 33%. It can be used to evaluate a PA level Q_o corresponding to the F_o level. Thus, the data in Fig. 2*A* indicates that the maximal variable thermal emission (Q_m –

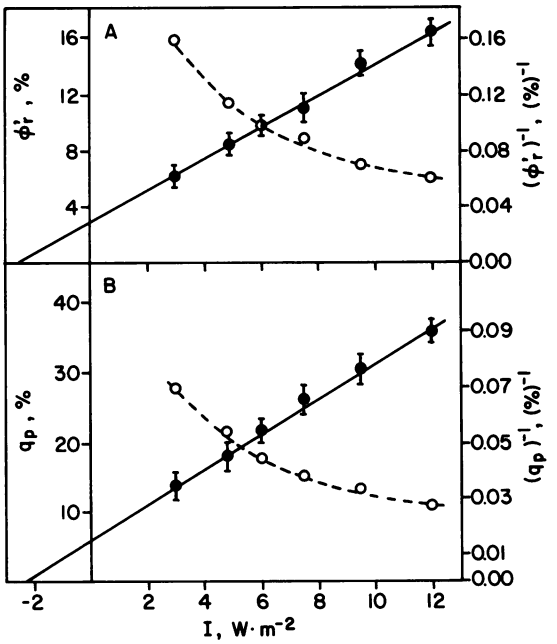

FIG. 2. Effect of the PA measuring beam intensity on ϕ_f' (*A*) and q_p (*B*) (○) and on the reciprocal of these parameters (●). The data were obtained from traces similar to Fig. 1 under aerobic conditions. The half-saturation value for the measuring beam was 2.4 W·m^{-2}. The values for ϕ_f' and q_p at $I = 0$ are 33% and 67%, respectively.

Q_o) represents about 33% of the maximal thermal emission (Q_m). A similar treatment can be applied to the fluorescence measurements (Fig. 2*B*) to find that $F_m - F_o$ represents about 67% of the maximal fluorescence (F_m) as confirmed from the fluorescence trace in Fig. 1 (aerobic conditions). The level Q_o can also be evaluated from the PA signal (without the saturating background light) obtained with a sample treated with $K_3Fe(CN)_6$ or any other electron acceptor at a sufficiently elevated concentration that prevents the formation of F_v in the presence of the PA modulated light (results not shown).

The ratio Q_o/Q_m is thus 2 times greater than F_o/F_m. However, the similar light saturation behavior of ϕ_f' and q_p demonstrated by their identical half-saturation light intensity (I_{50} = 2.5 W·m^{-2}) found in Fig. 2 favors the hypothesis of a common origin for F_v and Q_v. The above hypothesis will be substantiated by the following experiments.

Open reaction centers are characterized by oxidized primary acceptors [P680 Pheo] Q_A. Closed reaction centers in the photochemically active form [P680 Pheo] Q_A^- can be generated by a low redox potential. In this state, the increased rate of charge recombination in the pair [P680$^+$ Pheo$^-$] leads to a high initial level of fluorescence (F_o) near or at F_m (Fig. 1) (18, 23). The presence of the PA measuring beam does not modify this state, and a PA signal at the level Q_m can be monitored (Fig. 1). However, addition of the strong background light converts the reaction centers to the photochemically inert state [P680 Pheo$^-$] Q_A^-, where the yield of charge separation in the primary photoreaction is strongly decreased as well as charge recombination and the corresponding luminescence (18, 19, 24). Thus, a strong fluorescence quenching is observed upon illumination by actinic light under reducing conditions, which is most effective under anaerobic conditions (Fig. 1). Most evidently, a similar quenching of thermal dissipation is also observed in Fig. 1. Such negative PA signals were previously recorded from intact leaves and were due to the contribution from modulated oxygen evolution (25). In the present case in which isolated membrane fragments are used, the oxygen component is negligible at the phase angle of the thermal signal (8, 26, 27). Besides, oxygen photoevolution is prevented because of prereduction of the PSII electron acceptors.

The correspondence between F_v and Q_v can be further demonstrated by using PSII preparations where Mn has been depleted from the oxygen-evolving complex or where Mn has been depleted together with Fe from the Q_A–Fe–Q_B complex. The first type of preparation is studied in Fig. 3. In Fig. 3 *Upper*, the expected reduction of variable fluorescence that results from Mn depletion from the oxygen-evolving complex is shown together with the recovery of variable fluorescence after reconstitution of the PSII preparations with Mn at a ratio of 10 Mn per reaction center. The effects of Mn depletion and reconstitution on the PA signals Q_m and Q_v (Fig. 3) are similar to the effects on the fluorescence levels F_m and F_v. In fact, in Fig. 3 *Lower*, the close relationships between ϕ_f' and q_p during reconstitution with various Mn/reaction center ratios are clearly demonstrated.

Our data show that under the conditions that are not favorable for the formation of the long-lived state(s) [P680$^+$ Pheo$^-$] in the presence of the 34-Hz modulated beam [oxidized Q_A, inhibition of Q_A photoreduction by Mn removal or addition of $K_3Fe(CN)_6$, and photoaccumulation of the state [P680 Pheo$^-$] Q_A^{2-}], thermal emission is significantly decreased. This indicates that the decay of the pair [P680$^+$ Pheo$^-$] may be responsible for the variable thermal dissipation measured in our experiments.

The samples simultaneously depleted in Mn and Fe are studied in Fig. 4, where they are compared with a control, untreated sample. The depletion produces samples with a relatively high F_o level but no variable fluorescence was obtained. Similarly, there was no variable thermal dissipation in these samples. Addition of a saturating amount of Mn to reconstitute the Mn sites as well as the Fe sites (20, 22) did not significantly modify the thermal and fluorescences traces.

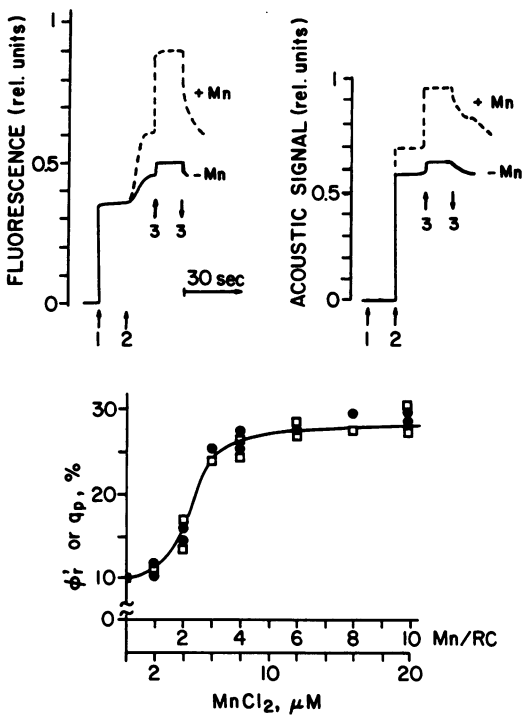

FIG. 3. (*Upper*) Traces obtained from preparations where the oxygen evolving complex has been depleted of Mn (−Mn) or reconstituted with Mn to a Mn^{2+}/reaction center ratio of 10 (+Mn). (*Lower*) Effect of the reconstitution of the Mn complex with various Mn/reaction center (RC) ratios on ϕ_f' (●) and q_p (□). Results from two separate experiments are presented. Conditions were as in Fig. 1 (aerobic conditions). rel; Relative.

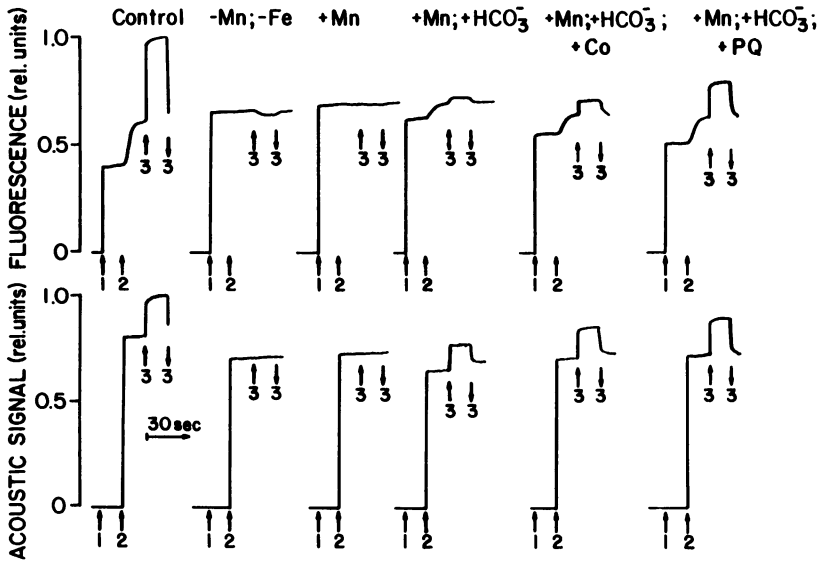

FIG. 4. Effect of the depletion of Mn from the oxygen evolving complex and Fe from the Q_A–Fe–Q_B complex (−Mn; −Fe) and subsequent sequential reconstitution with 100 μM Mn^{2+}, 2 mM HCO_3^-, 100 μM Co^{2+}, and 300 μM PQ (PQ-9). Conditions were similar to those in Fig. 1 (aerobic conditions). rel., Relative.

However, reconstitution with both Mn and HCO_3^- resulted in a partial recovery of the variable components of thermal and fluorescence emissions (Fig. 4). The role of HCO_3^- in restoring the variable emissions may be due to promotion of Mn and Co binding to the PSII complex or protonation of reduced Q_A and Q_B. It has been suggested previously that HCO_3^- could be involved in protonation of PSII quinone acceptors (28, 29). Protonation of reduced Q_A improves the yield of charge separation owing to the removal of the electrostatic repulsion between $Pheo^-$ and Q_A^- (30–32). Further, under these conditions, the F_o level found was intermediate between the level of F_o in open reaction centers and F_m in agreement with recent literature (32). A better recovery of variable fluorescence and variable thermal dissipation is obtained when Co is added together with Mn and HCO_3^- in the reconstitution medium (Fig. 4). In fact, Co is more effective than Mn in replacing Fe in the Q_A–Fe–Q_B complex (20, 22). It was suggested by Michel and Deisenhofer (33) that the Fe provides a binding site for HCO_3^-. Thus, it is possible that Co is a better ligand than Mn for HCO_3^-. On the other hand, it was also demonstrated that the formation of a complex between Q_A and Fe was required to form a stable primary acceptor complex (20).

The most effective reconstitution was obtained in the presence of PQ added together with Mn^{2+} and HCO_3^-, which could restore the Q_B site and improve the yield of variable fluorescence to nearly 50% of the initial value (Fig. 4). Variable thermal dissipation was apparently restored in a greater proportion. In fact, about 95% of the initial ϕ_f' was recovered under the best conditions, and 80% of ϕ_f' was restored in the presence of only Mn and HCO_3^- in the reconstitution medium. These experiments clearly show that the yield of variable thermal dissipation greatly depends on the presence of HCO_3^-. The above also demonstrates that in contrast to variable fluorescence which depends on the yield and stability of the primary charge separation, variable thermal dissipation emission also depends on another factor linked to the presence of HCO_3^- in the medium. To explain this apparent discrepancy, it must be considered that the enthalpy of reaction governs the amount of energy stored and therefore not released as heat. Entropic factors like conformational changes will directly influence the global thermal energy released by the system. Govindjee and Wasielewski (34) suggested that the function of HCO_3^- is linked with both protonation and conformational change events that influence the global free energy of the charge separation. The data presented here largely support this idea.

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