Insight into the relationship of chlorophyll a fluorescence yield to the concentration of its natural quenchers in oxygenic photosynthesis

(photosystem II/S states of oxygen-evolving complex/quinone acceptors/Spinacia oleracea)

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ABSTRACT  Fluorescence of chlorophyll a (Chla) is a noninvasive and very sensitive intrinsic probe of photosynthesis. It monitors the composition and organization of the photosystems, the exciton energy transfer, the photochemistry, and the effects of various types of stress on plants. It is the most used as well as the most abused tool in photosynthesis. Thus, an understanding of its relationship to photosynthesis has been of paramount importance. Both the oxidized primary plastoquinone, QA, and the oxidized primary reaction-center Chla, P680+ (for short, P*), are known to be quenchers of Chla fluorescence yield (ϕ) of photosystem II. Flash-number dependent of Chla fluorescence yield shows either a period 4, due to the four-step charge-accumulation process of water oxidation (donor side), or period 2 behavior, due to the two-step reduction of the plastoquinone Qb (acceptor side) of photosystem II reaction centers. We provide here a further insight into the relationship of variable Chla fluorescence yield (ϕ) to the concentration of the two quenchers. The observed time dependence of the ratio of ϕf after flash 3 to that after flash 1 (or flash 5) in spinach thylakoids at pH 6 can be explained if we suggest that 1/ϕf ≈ a[QA] + b[P*] + c, where a, b, and c are constants. From this it follows that the quenching of Chla fluorescence by P680+ after a flash is dependent on QA: for low [QA] (when most reaction centers are closed, [QA] is low) the quenching of Chla fluorescence by P680+ predominates, while for high [QA] (when most reaction centers are open), the quenching of Chla fluorescence is due predominantly to the increased concentration of the reduced form of P680 ([P*] is low).

The quantum yield of chlorophyll a (Chla) fluorescence (ϕ) is determined by the expression (see, e.g., refs. 1 and 2)

\[ ϕ = k_I/(k_I + k_2 + k_{phot}) \]  

where \( k_I \) is the rate constant of fluorescence, \( k_2 \) is the sum of the rate constants for radiationless deexcitation, excluding photosynthesis, and \( k_{phot} \) is the (pseudo)monomolecular rate constant of photosynthesis, which reflects photochemical utilization. In oxygenic photosynthesis, there are two photochemical reactions, labeled I and II, that operate in series (e.g., ref. 3). Photosystem II (PSII) oxidizes water and reduces plastoquinone, whereas photosystem I (PSI) oxidizes plastoquinol, via several intermediates, and reduces pyridine nucleotide, NADP++. It is the photochemistry of PSII, not that of PSI, that controls the \( ϕ_I \) of plants (e.g., ref. 2). The quenching of Chla fluorescence in photosynthesis was suggested (4) to be mainly determined by the oxidized form of the primary quinone acceptor (QA) of the PSII reaction center. Considering that \( k_{phot} = k_p[QA] \), we may rewrite Eq. 1 as

\[ ϕ = k_I/(k_I + k_2 + k_p[QA]) \]  

where \( k_p \) is the bimolecular rate constant for PSII photochemistry.

In PSII, light initiates the electron transfer from the primary Chla donor (P680) to the primary electron acceptor phophytin (Pheo), forming P680⁺ and Pheo⁻ (5). The latter transfers its electron to QA, which, when reduced, transfers it to the secondary quinone acceptor (Qb). Unlike QA, which is a one-electron carrier, Qb is a two-electron acceptor (see ref. 6). Plastoquinol (QbH₂), generated after a double turnover of PSI, then exchanges with an oxidized molecule of the plastoquinone pool (see review in ref. 7). This two-step process, also called the two-electron gate, leads to a period 2 oscillation in Chla fluorescence yield because electron flow from QA to Qb is faster that from QA to Qs (8). On the other hand, P680⁺, produced in the photochemical reaction of PSII, transfers its positive charge to a manganese complex (its redox state being labeled as S), via an intermediate, YZ (a tyrosine moiety). Here, however, four positive charges must accumulate before water is oxidized to molecular O₂. This leads to a periodicity of 4 in the O₂ evolution when measured as a function of flash number (e.g., ref. 9; reviews in refs. 10 and 11).

An increase in the Chla fluorescence yield after short flashes in the nanosecond to submicrosecond time range (12) suggested that P680⁺ is also a quencher of Chla fluorescence (13). To accommodate the quenching character of both P680⁺ and QA, Sonneveld et al. (14, 15) assumed that the most fluorescent state of PSII is PQ决定了 where P represents P680. With such an assumption, it was easy to explain the dependence of the rate of Chla fluorescence-yield changes upon the redox state of not only the acceptor side, but also the donor side, of PSI (16). Depending on the experimental conditions, one can observe, when plants are exposed to a series of flashes, a periodicity of 4 in the fast (microsecond) (17, 18) or in the slow (millisecond and second) components of Chla fluorescence yield (18, 19) as affected by the four-step charge-accumulating process in the oxygen-evolving complex. The flash number-dependent period 4 oscillation in the Chla fluorescence yield is explained by the different rate of

Abbreviations: Chl, chlorophyll; PSII, photosystem II; P680 (or P), electron donor of PSII; QA, primary one-electron quinone acceptor of PSII; Qb, secondary two-electron quinone acceptor; Ss, charge-accumulating state on the donor side of PSII (where k is 0, 1, 2, 3, or 4); YZ, a tyrosine of PSI that is the electron donor to P680⁺.

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reduction of the quencher P680+ by Y2, which is dependent upon the redox state of the water oxidation complex, the S states.

In this paper, we analyzed the changes in Chla fluorescence yield that occur from 70 μs to 10 ms after actinic flashes in spinach thylakoids at pH 6. In view of the two-step reduction of plastoquinone discussed above, alternate flashes would place the electron acceptor side in the same status. Thus to diminish contributions of the acceptor side, we used the ratios of the Chla fluorescence yields after flashes 1, 3, and 5 (flash 3/flash 1 and flash 3/flash 5). Analysis of such data in terms of the proposed relationship \(1/\psi = a[Q_A] + b[Q_A]^2 + c\) (where \([Q_A]_0\) is the normalized concentration of the open traps, \([P^\ast]\) is the normalized concentration of P680+, and \(a, b, c\) are constants) leads us to conclude that the quenching of \(\psi_f\) by P680+ is dependent upon the concentration of \(Q_A\): for low \([Q_A]\), quenching increases with increased \([P680^\ast]\), but for high \([Q_A]\), quenching increases with decreased \([P680^\ast]\).

**MATERIALS AND METHODS**

Spinach (Spinacia oleracea) thylakoids were isolated as described (20). The reaction medium contained 0.4 M sorbitol, 50 mM NaCl, 2 mM MgCl2, and 20 mM Mes (pH 6). The concentration of Chl in the sample was 10 μM. When used, benzoquinone was at 40 μM. All the additions were made in the dark.

Chla fluorescence yields after single-turnover saturating flashes were measured as described (21). Measurements of relative fluorescence yield, at different times after a flash, were made on 10-min-dark-adapted spinach thylakoid suspensions. The initial fluorescence intensity, \(F_o\), was measured with a weak (exciting only 1% of reaction centers), short (2.5 μs), blue (CS 4-96 Corning glass) xenon flash. Decays of relative fluorescence yield, after single-turnover saturating blue flashes, were then measured from 70 μs to 10 ms, also with weak flashes, used previously to monitor \(F_o\). The data were plotted as \((F - F_o)/F_o\). This is nothing else but the variable Chla fluorescence yield, normalized to the yield of “constant” fluorescence. The latter is proportional to the yield prior to photochemistry (when \([Q_A] = 1\), the maximum) and is emitted in competition only with the excitation energy transfer to the reaction center (16).

**RESULTS AND DISCUSSION**

**Kinetics of Chla Fluorescence Induced by the First, Third, and Fifth flashes.** Fig. 1 shows the kinetics of Chla fluorescence of spinach thylakoids at pH 6 induced by the first, the third, and the fifth flash, in the absence (A) or presence (B) of 40 μM benzoquinone. Benzoquinone was added to ensure that the quinone acceptor complex of the reaction center of PSII was mostly in the \(Q_AQ_B\) state (22, 23). Thus, subsequent flashes of light were expected to induce the binary oscillation of the quinone acceptor complex, producing the semiquinone form of \(Q_B\) after the first, third, etc. flashes. Our results on Chla fluorescence-yield decay (Fig. 1, compare A and B) show that in our samples the addition of benzoquinone did not make any significant difference. Thus, in our samples PSII was mostly in the \(Q_AQ_B\) state.

Fig. 2 shows the ratio of the relative Chla fluorescence yields induced by the third and the first as well as by the third and the fifth flash for spinach thylakoids in the absence (A) or presence (B) of 40 μM benzoquinone. These ratios increased with a characteristic time of about 0.3 ms and decreased with a characteristic time of about 2 ms. To understand the observed time dependence of these ratios we will first discuss the well-known view of the relationship of

**Quantum Yield of Chla Fluorescence: The Well-Known View.** The quantum yield of Chla fluorescence can be described by an extension of Eq. 1, the classical Stern-Volmer equation for PSII (24):

\[
\psi = k_f/(k_f + k_p[Q_A]).
\]

When the reaction centers are open—i.e., when \([Q_A]\) is maximal—the relatively high concentration of reaction centers in the state \(Q_A\) is responsible for the decrease of fluorescence yield. However, Eq. 3 does not include the well-known quenching of Chla fluorescence by P680+ (13). To resolve this problem, Duyssens and coworkers (14, 15, 24) have assumed that the relative Chla fluorescence yield, \(\psi_r\), is proportional to the relative concentration of state \(Q_A\) (also see ref. 25):

\[
\psi_r \propto [Q_A].
\]

This relationship qualitatively describes the Chla fluorescence quenching by both P680+ (since more P means less P+ and vice versa) and \(Q_A\).

From Eq. 4, and the fact that after alternate flashes, the electron acceptor side is almost identical, it follows that the ratio of Chla fluorescence yield induced by the third \((\psi_r(3))\) and first \((\psi_r(1))\) flashes (as well as the third and fifth flashes) will mainly depend on the donor side of the reaction center.
\[ \psi_f(3)/\psi_f(1) = P_3(t)/P_1(t) \]
\[ \psi_f(3)/\psi_f(5) = P_3(t)/P_5(t), \]

where \( P_k(t) \) is the concentration of the reduced form of P680 at time \( t \) after the \( k \)th flash, normalized to the reaction-center concentration.

Eq. 5 shows that the ratio \( \psi_f(3)/\psi_f(1) \) in the microsecond to millisecond time scale must be determined only by the kinetics of the P680+ reduction as affected by the four-step charge-accumulating S states.

The kinetics of P680+ reduction after a flash in the microsecond to millisecond time domain is determined by a sequence of the monomolecular transitions: \( S_1 Y_Z P^+ \rightleftharpoons S_1 Y_Z^+ P \rightleftharpoons S_{k+1} Y_Z P \). The transition \( S_1 Y_Z P^+ \rightleftharpoons S_1 Y_Z P \) occurred in the submicrosecond time domain, while the transition \( S_1 Y_Z P \rightleftharpoons S_{k+1} Y_Z P \) occurs in the micro- to millisecond time domain. Hence, the fast component of the P680+ reduction (due to the reaction \( S_1 Y_Z P^+ \rightleftharpoons S_1 Y_Z P \)) is out of the time resolution of our experiments, but we can consider the following kinetics by assuming that a quasi-equilibrium is reached in this reaction. Reduction of \( Y_Z \) in the reaction \( S_1 Y_Z P \rightleftharpoons S_{k+1} Y_Z P \) will "force" the further reduction of \( P^+ \). Only this slow component of \( P^+ \) reduction could be responsible for the period 4 modulation of Chla fluorescence yield considering below. The times of the \( S \) transitions are known to be approximately 30, 100, 300, and 1000 μs for \( S_0 \rightarrow S_1, S_1 \rightarrow S_2, S_2 \rightarrow S_3 \), and \( S_3 \rightarrow S_4 \) transitions, respectively (e.g., ref. 26).

Each \( P_k(t) \) in Eq. 5 must be an increasing function of time due to the stabilization, through the \( S \) states, of the reduced form of P680 in the microsecond time domain. This means that the ratios in Eq. 5 must increase with a characteristic time of about 1000 μs (numerator in Eq. 5), corresponding to the reduction of P680+ through the \( S_1 \) state, and decrease with a characteristic time of about 100 μs (denominator in Eq. 5), corresponding to the reduction of P680+ through the \( S_3 \) state. In contrast to this prediction, the experimentally measured ratios (Fig. 2) decrease in the millisecond time range. Even if one considers that, in dark-adapted samples, the initial state of the donor side of PSI I is a mixture of 75% \( S_1 \) and 25% \( S_0 \) states of the oxygen-evolving complex (9, 10), the same contradictory behavior persists. This is the observation that led to a further understanding of the factors which control Chla fluorescence yield.

\[ \phi_f = k_f/(k_t + k_g + k_{PQA} + k_{P+}). \]

Here, the concentration of reaction centers in the state [PQA] is responsible for the decrease of fluorescence when the reaction centers are open, and \( k_q \) is the rate constant of quenching of Chla fluorescence by P680+.

Interestingly, an increase in [P+] in Eq. 6 produces two opposite effects: it decreases the number of open reaction centers (the term \( k_g [PQA] \)), and thus increases Chla fluorescence yield, while it decreases Chla fluorescence yield as \( P^+ \) is a quencher. The sum of these two effects will be determined by the values of the rate constants \( k_p \) and \( k_g \) as well as by [QA] concentration.

If \([QA] = 0 \) (most reaction centers are closed), \( \phi_f \) is mainly determined by [P+]:

\[ \phi_f = k_f/(k_t + k_g + k_{P+}). \]

That is, P+ quenches fluorescence, in agreement with experiments of Mauzerall (12) and Sonneveld et al. (14).

If, however, \([QA] \approx 1 \) and \( k_p > k_g \), then \( \phi_f \) is mainly determined by the concentration of open reaction centers ([P]+ [P+] = 1):

\[ \phi_f = k_f/(k_t + k_g + k_q + [P][P+] + k_{P+}). \]

That is, the quenching of Chla fluorescence is mainly determined by the concentration of the reduced form of P680.

Fig. 3 shows the dependence of quantum yield of variable Chla fluorescence (\( \psi_f \)) on P680+ concentration calculated on the basis of Eq. 3 (A), Eq. 4 (B), and Eq. 6 (C) with the assumption that [PQA] = [P][QA]. In the case of the Stern-
Volmer equation, Eq. 3, $\psi_f$ increases with increasing [P680+] for all QA concentrations (Fig. 3A); i.e., the reduced form of P680 is the quencher of Chl$\alpha$ fluorescence. This disagrees with the quenching nature of P680$^+$. In the case of Eq. 4, $\psi_f$ is a linearly decreasing function of [P680$^+$] for almost all QA concentrations (Fig. 3B); i.e., the oxidized form of P680 is the quencher of Chl$\alpha$ fluorescence. Eq. 6 predicts that when [QA] is very low, P$^+$ approximately linearly quenches fluorescence, whereas when most traps are open ([QA] $\approx$ 1), the reduced form of P680 begins to act as a quencher of Chl$\alpha$ fluorescence; i.e., $\psi_f$ increases with increasing [P680+] (Fig. 3C). When $k_q = k_p$, Eq. 6 reduces to $\psi_f = k_f/(k_f + k_p + k_p(1 - [PQ_A]^2))$, which predicts the increase of $\psi_f$ with [PQ$\lambda$], similar to Eq. 4.

Fig. 4 shows the values of the ratios $\psi_f(3)/\psi_f(1)$ and $\psi_f(5)/\psi_f(4)$ calculated from two different approaches (Eqs. 4 and 6). The dashed line in Fig. 4 shows the prediction, from Eq. 4, that has usually been considered to be satisfactory. However, the prediction shows a rise in Chl$\alpha$ fluorescence yield in the millisecond time scale, whereas the actual data show a decline in Chl$\alpha$ fluorescence yield (Fig. 2). The absence of fit with the experimental results clearly points out that this relationship is insufficient to explain the experimental data. The calculation based on the generalized Stern–Volmer equation (solid line, Eq. 6) is, however, in good qualitative agreement with that obtained experimentally (Fig. 2), indicating reasonable approximation of the quantum yield of Chl$\alpha$ fluorescence by the relationship suggested in this paper.

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