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REVIEW

Photochemical and thermal phases of chlorophyll *a* fluorescence

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Abstract

The measurement of variable chlorophyll (Chl) *a* fluorescence is widely used as a convenient and versatile tool in photosynthesis research. In many applications empirical correlations and simplified models of Chl *a* fluorescence are used with success. Nevertheless, variable Chl *a* fluorescence provides only indirect and complex image of processes occurring within photosynthetic membranes and such simplifications have only limited validity. In this review we elucidate some controversial and still unresolved questions about the origin and interpretation of the variable Chl *a* fluorescence induction and the proper use of variable Chl *a* fluorescence for studies of photochemical events in photosystem 2 (PS2). Although

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Abbreviations: Chl - chlorophyll; cyt - cytochrome; D1 - protein, *psbA* gene product; DCMU - 3-(3,4-dichlorophenyl)-1,1-dimethylurea; F_m - maximal fluorescence yield; F_0 - intrinsic (minimal) fluorescence yield; F_v - variable Chl *a* fluorescence yield, $F_v = F_m - F_0$; MTF - mulitple turnover flash; P680 - primary electron donor of photosystem 2; Pheo - pheophytin; PPFD - photosynthetically active photon flux density; PQ - plastoquinone-9; PS - photosystem; Q_A (Q_B) - primary (secondary) quinone acceptor of PS2; q_N - nonphotochemical quenching coefficient; q_P - photochemical quenching coefficient; RC - reaction center; STF - single turnover flash (typically of ~µs duration); Tyr_Z - redox-active tyrozine in PS2; ΔpH - transthylakoid proton gradient; Φ_{O2max} - maximal yield of oxygen evolution; Φ_{PS2max} - maximal photochemical yield of photosystem 2.

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the major part of variable Chl *a* fluorescence reflects the photochemical closure of the PS2 reaction centers (RCs) and can be considered as *a* function of the redox state of the primary acceptor Q_A, up to 50 % of the change in the Chl *a* fluorescence yield can be of secondary, nonphotochemical origin. We review the possible sources of the inherent heterogeneity in the origin of variable Chl *a* fluorescence. We also comment on the practical implications this bears for the use of variable Chl *a* fluorescence.

Additional key words: photochemical and nonphotochemical quenching; photosystem 2; Q_A; reaction center.

Introduction

The recent progress in techniques of Chl *a* fluorescence measurements has made significant impact on photosynthesis research. Chl *a* fluorescence is routinely used in laboratory and field studies as a fast, noninvasive, and relatively simple tool to measure the efficiency and kinetics of photosynthetic processes that involve PS2. Attempts are made to extend the scope of the method far beyond PS2. For example, Chl *a* fluorescence is used to estimate photosynthetic electron transport rates and productivity of whole plants and ecosystems (Kolber and Falkowski 1993, Ball *et al.* 1995), to check plant responses to various abiotic (Neuner and Buchner 1999) and biotic (Balachandran *et al.* 1997) stresses, to detect side-effects of mutations in plants (Voigt *et al.* 1997), to assess physiological damages of fruits and vegetables during post-harvest storage (DeEll and Toivonen 1999), to predict seed quality (Jalink *et al.* 1998), *etc.* The recent boom in the use of Chl *a* fluorescence results mostly from the progress in instrumentation: improved electronics allows the construction and production of more versatile and sensitive fluorometers with features than could be only dreamed of several years ago (*e.g.*, Kolber *et al.* 1998, Schreiber 1998, Nedbal *et al.* 1999). However, the technological progress and general availability of efficient instruments have not been paralleled by similar increase in the knowledge of Chl *a* fluorescence. In many cases, the relations between variable Chl *a* fluorescence to functional and structural events in PS2 and to the efficiency of photosynthetic electron transport are semi-empirical. Although the validity of such empirical concepts has been questioned (Holzwarth 1993, Falkowski *et al.* 1994) and new theoretical models for variable Chl *a* fluorescence were presented (Trissl *et al.* 1993, Lavergne and Trissl 1995, Stirbet *et al.* 1998, Lazar 1999), the imbalance between the large popularity and potential of the Chl *a* fluorescence on one hand and the poor understanding of some basic mechanisms underlying its interpretation on the other hand is persistent.

The discussions at the 2nd Regional Photosynthesis Workshop (held at Lipno, Czech Republic, January 1999) aimed to sum up the current knowledge on Chl *a* fluorescence and pointed out several controversial problems and open questions that remain to be resolved. One of these open questions concerns the basic assumption underlying the interpretation of Chl *a* fluorescence. The variable Chl *a* fluorescence induced by saturating irradiation reflects the photochemical reaction in PS2 RCs, in agreement with the early hypothesis of Duysens and Sweers (1963) stating that the redox state of the primary quinone electron acceptor Q_A is the main factor controlling the yield of variable Chl *a* fluorescence. In dark-adapted and intact samples, all Q_A molecules are oxidized (open PS2 RCs) and the Chl *a* fluorescence yield is minimal; upon a saturating flash, all Q_A molecules become transiently reduced (closed PS2 RCs), and the Chl *a* fluorescence yield is maximal. This interpretation prevails for more than three decades, but its validity turned out to be one of the most debated topics. There is now a large body of evidences indicating that the variable Chl *a* fluorescence is composed of two distinct phases of approximately equal amplitudes: a photochemical phase related to the redox state of the primary quinone accceptor Q_A of PS2 and a thermal or nonphotochemical phase whose origin is still unclear (Samson and Bruce 1996, Schreiber and Krieger 1996).

In this review we discuss the intrinsic heterogeneity of variable Chl *a* fluorescence. This heterogeneity must not be confused with the effects of PS2 antenna and PS2 reducing side heterogeneities on variable Chl *a* fluorescence (Govindjee 1990, Melis 1991, Lavergne and Briantais 1996). We present the experimental evidences leading to the distinction between the two phases of variable Chl *a* fluorescence, their origins, and their respective significance for the assessment of electron transport efficiency and the integrity of PS2 complexes. The reader will therefore not find here a complete and comprehensive review of variable Chl *a* fluorescence phenomena. For this, the reader should refer to other review articles (Krause and Weis 1991, Dau 1994a, Evans and Brown 1994, Govindjee 1995, Mohammed *et al.* 1995, Pospíšil 1997, Lazar 1999).

Presence of two distinct types of variable Chl *a* fluorescence

Within few years after the publication of the single fluorescence quencher hypothesis by Duysens and Sweers (1963) the presence of two distinct phases of Chl *a* fluorescence was demonstrated by Morin (1964) and Delosme (1967). Upon a sudden irradiation of dark-adapted green algae or isolated chloroplasts with a very strong actinic source (maximum irradiance reached in few µs) they observed a rapid rise of variable Chl fluorescence followed by a slower rise of a similar amplitude. They established that the rate of Chl *a* fluorescence rise during the fast phase was determined by the number of photons absorbed whereas the slow phase became independent to irradiance beyond saturation. Their observations were not reproduced by other laboratories for the next twenty years probably due to the hazards of the experimental set-up: for a very rapid irradiation of the sample with an intense actinic source, a bullet was fired from a *Anschutz 22* long rifle to expel a metallic screen placed between the sample and the radiation source! Later, by using an array of light-emitting diodes for fast and intense irradiation, similar observations were made by Neubauer and Schreiber (1987). They confirmed the photochemical and thermal natures of the fast and slow phases, respectively, from their dependencies toward irradiance and also from their dependencies toward temperatures: the fast phase was temperature-insensitive whereas the slow phase was abolished at subfreezing temperatures. However, the slow phase was practically unaffected by temperatures between 10 and 25 °C, indicating that this phase is not directly controlled by temperature (Neubauer and Schreiber 1987). In addition, Strasser *et al.* (1995) using the commercial fluorometer 'Plant Efficiency Analyser' (PEA) from *Hansatech* (UK), were able to measure accurately the kinetics between F_0 and F_m which exhibits usually three steps called J, I, and P. The thermal phase is composed of two subphases, the first one observed in the range of ms to tens of ms and the second one seen from tens to hundreds of ms. However, in light-adapted materials, the distinction between these two subphases vanished at rather low irradiances (Schreiber *et al.* 1995a).

Besides the observations made by Delosme (1967) and Neubauer and Schreiber (1987), the photochemical nature of the fast Chl *a* fluorescence phase is further supported by observations showing that the yield of variable Chl *a* fluorescence induced by a single turnover flash (STF) of saturating energy does not exceed the amplitude of the fast phase of variable Chl *a* fluorescence (up to 50 % of total variable Chl *a* fluorescence) induced by an intense actinic radiation (Schreiber *et al.* 1986, 1995b, Samson and Bruce 1996). In addition, Joliot and Joliot (1979) demonstrated from spectroscopic measurements that about 90 % of all the Q_A molecules were reduced after one saturating STF. Therefore, the fast Chl *a* fluorescence phase may be determined by the rate of Q_A photoreduction and also practically all the Q_A molecules are reduced at the completion of the fast fluorescence phase (Delosme 1967, Neubauer and Schreiber 1987).

Possible origins of the thermal phase of Chl *a* fluorescence

Contrary to the photochemical phase of variable Chl *a* fluorescence, the exact origins of the thermal phase are still unclear. Several factors could quench the Chl *a* fluorescence yield measured when all the Q_A molecules are reduced following a saturating STF or after 1 to 2 ms under a very strong continuous irradiation. Then the kinetics of the thermal phase reflects the reduction of the PQ pool (Delosme 1967, Srivastava *et al.* 1995). The presence of oxidized PQ molecules therefore appears somehow related to much of the thermal phase of Chl *a* fluorescence (Kramer *et al.* 1995). This is consistent with the high Chl *a* fluorescence yield induced by STF in spinach thylakoids resuspended in presence of duroquinol and sodium dithionite (short treatment of 15 s). As judged by their effects on F_0 and on the kinetics of Q_A^- reoxidation, these chemicals reduced most of the PQ pool and redox components of similar midpoint potential Em but had a small reducing effect on Q_A (Samson and Bruce 1996). In PS2-enriched membranes prepared with the detergent deoxycholate where Q_A was maintained in a reduced state, the main quencher of Chl *a* fluorescence was the photosynthetically active pool of oxidized plastoquinone (Van Gorkom *et al.* 1974). Its photoreduction was accompanied by a shift of a Chl *a* absorption band near 680 nm, suggesting the presence of some physical interaction between Chl *a* and PQ molecules. The quenching of Chl *a* fluorescence by oxidized PQ would be similar to the quenching effect of artificial quinones observed in solution and in chloroplasts. In solution, substituted quinones directly interact with excited states of Chls (Amesz and Fork 1967, Lee *et al.* 1992, Vasil'ev *et al.* 1998).

The presence of an oxidized PQ pool decreases Chl *a* fluorescence yields by 5-20 % (Wraight *et al.* 1972, Joliot and Joliot 1973, Van Gorkom *et al.* 1974, Vernotte *et al.* 1979). However, this quenching is variable and generally much smaller than the amplitude of the thermal fluorescence phase (Krause and Weis 1991, Schreiber and Krieger 1996). To explain the small Chl *a* fluorescence quenching effect of the oxidized PQ in presence of DCMU, we have to consider the presence of a possible intermediate controlling the quenching effect of the PQ pool on Chl *a* fluorescence. Initially, Delosme (1967) attributed the thermal phase of Chl *a* fluorescence to the "destruction of a quencher R associated with a pool of oxidants located between the two photosystems", *i.e.*, the PQ pool. The nature of R was not specified and was interpreted less as real quencher with a distinct molecular identity than a special interaction between the PQ molecules and the Chl *a* molecules associated with the PS2 complexes (see Etienne and Lavergne 1972). This interaction is modified in presence of inhibitors like *o*-phenantroline and DCMU that bind at the Q_B site of PS2, thereby blocking the electron transfer from Q_A^- to Q_B: these inhibitors increase the photochemical phase at the expense of the thermal phase (Delosme 1967, Etienne and Lavergne 1972, Neubauer and Schreiber 1987). It is important to point out the common feature of PS2 complexes in presence of Q_B-inhibitors and in presence of reduced PQ pool, *i.e.*, the absence of oxidized PQ molecules bound to the Q_B site. Therefore, an interesting hypothesis would be that the degree of occupancy of the Q_B site by oxidized PQ determines the Chl *a* fluorescence yield measured following the completion of the photochemical phase. This hypothesis is supported by results obtained with mutants of green alga *Chlamydomonas*. Series of mutations of a single amino acid in the Q_B binding site (Alanine 251 of the D1 protein) was created. The mutations had a distinct effect on the binding and exchange of PQ molecules in the Q_B pocket. While the variable Chl *a* fluorescence induced by single turnover flashes (photochemical phase) was comparable in all the mutants, the extent of thermal phase, induced by multiple turnover irradiation correlated with the rate of PQ binding in the pocket (O. Prášil, Z. Kolber, and P. Falkowski, unpublished). This would imply that the interaction of oxidized PQ with the antenna pigment bed is somehow localized in the vicinity of the Q_B binding site.

An important characteristic of the inhibition of the Chl *a* fluorescence thermal phase by DCMU is that it requires several STFs to be completed: in DCMU-treated chloroplasts the first saturating STF can induce only ~70-75 % of the total variable Chl *a* fluorescence (F_V), whereas a train of several flashes is needed to remove the remaining quenching (Joliot and Joliot 1977, 1979, Samson and Bruce 1996). Spectroscopic measurements indicated that about 90 and 97 % of the Q_A were reduced after the first and the second flashes, respectively (Joliot and Joliot 1981). These observations led Joliot and Joliot (1977, 1979, 1981) to propose the presence of two quenchers Q1 and Q2 that accept electron prior the DCMU inhibitory site. They showed that only Q1 is involved in active PS2 photochemistry corresponding to specific absorbance changes near 320, 515, and 550 nm. The Q2 quencher is not a plastoquinone and its reduction does not induce an electric field. Q2 was suggested to be part of an alternative electron pathway acting with a very low quantum efficiency. Despite enormous progress in our understanding of the PS2 structure and its redox components in the last two decades, the nature of Q2 has remained elusive and it appears to be a manifestation of the slow thermal phase. Compatible with this idea are the observations showing that dithionite reduces more rapidly the Q2 than the Q1 (Joliot and Joliot 1972) and also that dithionite eliminates faster the thermal phase compared to the photochemical part of Chl *a* fluorescence (Samson and Bruce 1996).

Another characteristic of the thermal fluorescence phase that may give a clue about its origin is the increase of Chl *a* fluorescence yield observed from 20 µs up to 1 or 2 ms following a STF given to DCMU-poisoned chloroplasts (Joliot and Joliot 1977, Schreiber and Krieger 1996). Joliot and Joliot (1977) reported that higher flash energy was required to induce the slower fluorescence rise from 20 µs to 2 ms compared to the fast fluorescence rise. They interpreted their results by the existence of a double-hit process involving the two electron acceptors Q1 and Q2 as discussed above. A close relation can be made between the Chl *a* fluorescence increase following a STF in DCMU-treated chloroplasts with observations showing that flash durations longer than 50 µs are required to increase the Chl *a* fluorescence yield above those measured with an STF (Valkunas *et al.* 1991, France *et al.* 1992). To explain the effect of flash duration on Chl *a* fluorescence yield, these authors proposed a sequential hit model, which also assumes a double-hit process as in the Joliot and Joliot's model: after the first photoreaction, the PS2 RCs in a low fluorescent state (Q_1) evolve in the dark within 50 µs to a different state (Q_2). Then, the second photoreaction will generate a high fluorescence state (Q_3). The nature of the putative intermediate states is still unknown. The flash duration influences also the shape of the Chl *a* fluorescence induction curve: for pulses shorter than ~2 µs (inducing only Q_1 state) the induction curves (measured by pump-probe technique, Mauzerall 1972) were exponential with no connectivity (Mauzerall 1976, Joliot and Joliot 1977, Strasser 1978, Deprez *et al.* 1983, Falkowski *et al.* 1986, Ley and Mauzerall 1986, Mauzerall and Greenbaum 1989, Valkunas *et al.* 1991, France *et al.* 1992); for actinic pulses of 2-50 µs duration, nearly exponential Chl *a* fluorescence curves with moderate connectivity p ~0.3 were obtained; and sigmoidal curves with high connectivity p ~0.55 were induced during multiple turnover excitation (>50 µs, Q_3 reduced) (Valkunas *et al.* 1991, France *et al.* 1992). This has also profound impact on the relation between variable Chl *a* fluorescence and active PS2 RCs: such relation seems to be linear for (sub)µs actinic flashes that induce only the photochemical phase of Chl *a* fluorescence induction and non-linear for continuous, multiple-turnover excitations (discussed in Jursinic and Dennenberg 1993). As a consequence, it is questionable whether the degree of connectivity between PS2 RCs (*e.g.*, Joliot and Joliot 1964, Joliot *et al.* 1971, Lavergne and Leci 1993, reviewed in Lazar 1999) can be reliably derived from the shape of Chl *a* fluorescence rise curve induced by multiple turnover pulses. For theoretical description of exciton dynamics in different models of photosynthetic antenna systems see Bernhardt and Trissl (1999).

An alternative view is that unlike the photochemical phase that is a function of formation of the closed state of PS2 (reduced Q_A), the thermal phase might not reflect change in the redox state of any specific molecule within photosynthetic membrane. Instead, the increase in Chl *a* fluorescence yields can be caused by transient changes in rates of competing dissipative pathways. In other words, the rates of non-radiative heat dissipation might be different for PS2 that are in the dark or excited by a single turnover flash and for PS2 exposed to multiple turnover excitation. The differences in heat dissipation rates could be caused, *e.g.*, by small conformational changes of PS2 related to binding of the secondary PQ acceptors (Reifarth and Renger 1998), or by local electrical fields formed by charge separation (Vredenberg *et al.* 1998).

Similar hypothesis for the origin of the thermal phase, based on the reversible radical pair model of PS2 photochemistry has been proposed by Schreiber and Krieger (1996). They suggest that a significant fraction of variable Chl *a* fluorescence results from enhanced recombination of the radical pair $P680^+Pheo^-$ when Q_A is reduced. This recombination Chl *a* fluorescence would compete with nonradiative energy loss at the RC whose rate constants, due to some changes in the RC (electrostatic field, charge stabilization, *etc.*) would decrease within 1 ms with a half-time of 50 μs. The changes of these rate constants would then be mirrored by an increase of the recombination Chl *a* fluorescence, resulting in the rise of the fluorescence thermal phase.

The different models mentioned above suggest that changes occurring in the PS2 RCs after the primary photochemical reaction could be responsible for the thermal fluorescence phase. However, chemical reduction of the PQ pool in spinach thylakoid by duroquinol (Em = +5 mV) or by dithionite (short incubation of 15 s) increases both the Chl *a* fluorescence yield induced by STF and the optical absorption cross-section of PS2 RCs determined from the flash saturation curve of variable Chl *a* fluorescence (Samson and Bruce 1996). Although the increases of PS2 cross-sections were markedly smaller in presence of DCMU, these results rather suggest that the oxidized PQ molecules somehow cause a non-photochemical quenching in the antenna pigment bed by a mechanism occurring before the primary photochemical reaction in PS2 complexes. This was elegantly demonstrated in a picosecond time-resolved study of Chl *a* fluorescence decay kinetics measured in spinach thylakoids immediately after either a saturating STF or a multiturnover flash (Vasil'ev *et al.* 1998). The Chl *a* fluorescence decay kinetics based on an exciton radical pair equilibrium showed that the low Chl *a* fluorescence yield measured after a STF could be well modeled by changing only the rate constant for nonradiative energy loss in the antenna of PS2. Chl *a* fluorescence decay kinetics of the low fluorescence yield induced by STF could not be satisfactorily described by the low yield of recombination Chl *a* fluorescence, as previously suggested (Schreiber and Krieger 1996). An antenna-based origin for the low Chl *a* fluorescence yield after the STF was experimentally supported by the observations showing that the decay kinetics and their modeled parameters of Chl *a* fluorescence induced by MTF in thylakoids resuspended in presence of the artificial antenna quencher, 5-hydroxy-1,4-naphthoquinone, were very similar to the decay kinetics of Chl *a* fluorescence induced by STF in the absence of added quinone.

It may seem illogical that the efficiency of open PS2 RCs to utilize excitation energy decreases when the PQ pool is mostly oxidized in conditions such as low irradiance, whereas this efficiency would increase in conditions where the rate of PS2 photochemistry exceeds the capacity of the electron transport chain causing the reduction of the PQ pool. The PS2 complexes are prone to photoinhibition under these conditions (Prášil *et al.* 1992). The rate of PS2 photochemistry estimated from the rate of Q_A photoreduction is significantly decreased in presence of nonphotochemical quenching, q_N (Genty *et al.* 1990a). Therefore, the induction of q_N, namely the one related to the formation of zeaxanthin (Pfündel and Bilger 1994), can outweigh the effect of the redox state of the PQ pool on the quantum efficiency of PS2 photochemistry.

Finally, the relative extent of the thermal phase seems to depend on the origin and complexity of the photosynthetic material. The photochemical and thermal phases are also present in cyanobacteria although their respective contribution to total variable Chl *a* fluorescence may be largely different (Strasser *et al.* 1995). Such differences may be attributed to variation of the redox state of the PQ pool which is partly reduced due to chlororespiration (Fork and Herbert 1993, Schreiber *et al.* 1995a). In algal cells the extent of differences depends on the acclimation of organisms to irradiance. The cells that were grown on high irradiances show much smaller thermal phase than cells grown at low irradiances (O. Prášil, unpublished). Again, this might point to the complex mechanisms involving interaction between conformation of the PS2, RC, PQ molecules, and processes in PS2 antenna structures.

Other possible quenching processes

Beside the specific quenchers mentioned in the previous section, there is a number of other redox active molecules and processes within photosynthetic membrane that modulate Chl *a* fluorescence yields (see Kramer and Crofts 1996). Here we will briefly review only these quenching processes that can potentially compete with both the photochemical and the thermal phases of variable Chl *a* fluorescence induction (time scale from nanoseconds to tens of milliseconds). There are also other processes that induce significant changes in variable Chl *a* fluorescence, *e.g.*, the transmembrane proton gradient, deepoxidation of xanthophylls, photoinhibition or state-transitions (for reviews see Dau 1994b, Kramer and Crofts 1996). These diverse processes, grouped under the broad term "nonphotochemical quenching" operate on time scales of seconds and longer, and will not be discussed here. The presence of the multitude of different quenchers does not necessarily complicate the analysis of variable Chl *a* fluorescence. One can take advantage of the differences in the lifetimes, quantum yields of formation or relative magnitudes of the quenching effects, and use Chl *a* fluorescence as a specific tool to study these secondary processes (*e.g.*, Reifarth *et al.* 1997).

Chl *a* fluorescence yield depends on the number of oxidizing equivalents stored on the donor side of PS2 (Joliot *et al.* 1971, Zankel 1973, Strasser B.J. 1997) and can be best observed as period-four oscillations in Chl *a* fluorescence yields F_0 and F_M

when the sample is exposed to series of single-turnover saturating flashes. Generally, the yield of *open* RCs (F_0) is higher in states S_2 and S_3 than in states S_0 and S_1. For *closed* centers, the yield (F_M) is higher in centers during transitions from $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$ than during transitions $S_2 \rightarrow S_3$ or $S_3 \rightarrow S_0$ (Delosme 1971, Joliot *et al.* 1971). The extent of modulation by the PS2 donor side can be up to 15 % of the photochemical phase. The origin and mechanism of this "donor-side" quenching are not clear. Part of the phenomena could be explained as a quenching by Chl cation radical $P680^+$ that is formed during the primary charge separation in the PS2 RC. $P680^+$ is a strong quencher (Butler 1972, Den Haan *et al.* 1974, Sonneveld *et al.* 1979, Deprez *et al.* 1983). Although the reduction of $P680^+$ by Tyr_Z^+ is dominated by nanosecond kinetics (Eckert and Renger 1988), a small fraction of PS2 (~15-20 %) exhibits a $P680^+$ reduction with significantly slower kinetics (half-time ~35 μs). The extent of the slow fraction of $P680^+$ reduction depends on the redox state of the water oxidizing enzyme, and during flash irradiation of dark adapted samples oscillates with a period of four. The 35 μs phase of $P680^+$ reduction is maximal in states S_2 and S_3 (Schlodder *et al.* 1985). Several observations indicate that the donor side quenching cannot be simply explained as to reflect the S-state dependent changes in the equilibrium constant between $P680^+$ and the secondary donor Tyr_Z. Zankel (1973) observed that methylamine and ferricyanide significantly changed the Chl *a* fluorescence yield oscillation pattern without affecting the oscillatory behavior of oxygen evolution. The period four oscillations in the Chl *a* fluorescence yield therefore result from coupling with other processes on the donor side, such as proteolytic reactions or electron donation from competing pathways with different quenching properties (Christen *et al.* 1998). The extent of $P680^+$ quenching depends also on the integrity of the water oxidizing complex, on the redox state of Q_A (Shinkarev and Govindjee 1993), and on other factors that can change the functional and conformational state of PS2 (recombination of $P680^+$ with alternative acceptor, induction of cyclic electron flow around PS2) (Schreiber and Neubauer 1989, Bruce *et al.* 1997).

When closed RCs receive multiple excitation, the separated charges $P680^+$ $Pheo^-$ can not be stabilized and charge recombination occurs. Due to the process of spin dephasing the recombination can result in formation of triplet states. The Chl triplets are localized on P680 or on one of the neighboring chlorophylls. Carotenoid triplets are then formed within several nanoseconds by triplet-triplet energy transfer from Chl triplets (Mathis 1969, Kramer and Mathis 1980, Schodel *et al.* 1998). Both carotenoid and Chl triplets quench Chl *a* fluorescence (Zankel 1973, Mathis *et al.* 1979). Since triplet states decay with half-time of few microseconds (Renger and Wolff 1977), the significant formation and accumulation of triplets occurs only during the actinic pulse of very high intensity (pulsed laser or xenon flashlamp). The actual decay rate of triplets depends on the concentration of oxygen. To avoid the quenching by triplets, Chl *a* fluorescence experiments should be performed using actinic flashes of relatively low energies. For recent papers on Chl *a* fluorescence quenching by carotenoid triplets see Reifarth *et al.* (1997) and Schodel *et al.* (1998, 1999).

The reduced primary acceptor pheophytin ($Pheo^-$) is a strong Chl *a* fluorescence quencher (Klimov *et al.* 1977). Under most of the physiological conditions $Pheo^-$ is reoxidized within 300 ps by Q_A (Eckert *et al.* 1988) or, if the Q_A is already reduced then $Pheo^-$ rapidly recombines with $P680^+$. The quenching can be thus observed only when $Pheo^-$ is allowed to accumulate (*e.g.*, when irradiated in the presence of dithionite or under anaerobic conditions).

The oxidized non-heme iron (Fe^{3+}) located between Q_A and Q_B can influence Chl *a* fluorescence yields by forming a fast reoxidation pathway for Q_A^-. The electron is transferred from Q_A^- to Fe^{3+} with a halftime of <25 μs (Diner and Petrouleas 1987, Petrouleas and Diner 1987). This is 5 to 10 times faster than the 100-300 μs kinetics of electron flow from Q_A^- to Q_B (Robinson and Crofts 1983). Since the subsequent reoxidation of Fe^{2+} takes seconds (Diner and Petrouleas 1987) this pathway should not influence significantly Chl *a* fluorescence yields under repetitive or multiple turnover excitations, but has to be taken into account when dark-adapted samples are excited by single-turnover flashes.

Very little is known about the functional role of cytochrome (cyt) *b*-559 in the PS2 RC (Whitmarsh and Pakrasi 1996). There are only few reports about its possible influence on Chl *a* fluorescence yields. These reports are based on experiments done at low temperatures. It was proposed that the oxidized cyt *b*-559 quenches Chl *a* fluorescence at 77 K when it acts as a donor to $P680^+$ (Okayama and Butler 1972, Butler *et al.* 1973). However, according to Schweitzer and Brudvig (1997) the Chl *a* fluorescence quenching at low temperatures rather results from the formation of cation on the accessory chlorophyll Chl_Z than from the oxidation of cyt *b*-559. Thus the suggestion of Joliot and Joliot (1973) that oxidation of cyt *b*-559 is responsible for part of the thermal phase is probably not valid.

Practical importance of the photochemical and thermal phases of Chl *a* fluorescence for the assessment of electron transport

The distinction between the photochemical and the thermal phases of variable Chl *a* fluorescence can have important consequences for the study of photosynthetic electron transport, namely for the estimation of the maximal and operational quantum yields of PS2 photochemistry, and also for the estimation of the photochemical and nonphotochemical fluorescence quenching coefficients.

In dark-adapted material the maximum photochemical yield of PS2 (Φ_{PS2max}) is estimated from the F_v/F_m ratio (where $F_v = F_m - F_0$) according to the saturation pulse method (Schreiber *et al.* 1986) or the pump-probe method (Falkowski *et al.* 1986). Both methods provide comparable values of the minimum Chl *a* fluorescence yield F_0 measured either by a weak modulated (measuring) irradiation or by a non-actinic probe flash. However, the amplitude of F_v can differ by up to 50 % depending on whether only the photochemical phase is induced by a saturating single-turnover pump flash or both the photochemical and thermal phases are induced by a long (~1 s) saturating radiation pulse. As a result, the F_v/F_m ratio can vary from ~0.65 up to ~0.83 when measured with a short or a long saturating flash, respectively. There is still a debate concerning which type of flash should be used to induce the F_m level (Schreiber *et al.* 1995b, Kolber *et al.* 1998).

The determination of Φ_{PS2max} as well as the fluorescence quenching analysis rely on the assumption that the radiation pulses used to induce the F_m yield reduce Q_A in all the PS2 complexes (thus suppressing photochemical quenching) without affecting the nonphotochemical quenching (Schreiber *et al.* 1995b, Prasil *et al.* 1996). Intuitively, the saturating flash used to obtain F_m should affect only photochemical processes within PS2. If one assumes that the change of Chl *a* fluorescence yield *during* the multiple turnover irradiation reflects the removal of a non-photochemical quenching, then the lower value (~0.65) should reflect the Φ_{PS2max} which would be overestimated by the use of a long saturating flash.

However, the low values for Φ_{PS2max} obtained from STF fall short of what can be expected from the maximum quantum yields of photosynthesis estimated from the initial slopes of irradiance saturated curves of O_2 evolution. Mean value of 0.106±0.001 for Φ_{O2max} (measured by Björkman and Demmig 1987) averaged from a large number of diverse species corresponds, respectively, to 95 and 85 % of the maximum theoretical quantum yield for C_3 plants of 0.111 and 0.125 assuming the absence or the presence of the Q-cycle (Long *et al.* 1993).

The discrepancy between the estimated Φ_{PS2max} and Φ_{O2max} results partly from the contribution of the constant PS1 Chl *a* fluorescence to the F_0 level at wavelengths greater than 700 nm, as typically measured with commercial modulated fluorometers (Genty *et al.* 1990b, Pfündel 1998). Both studies agree that about 30 and 50 % of the typical F_0 measurements in C_3 and C_4 plants, respectively, originate from PS1 fluorescence emission. Measurements of Chl *a* fluorescence at wavelengths near 690 nm would therefore minimize this artifact. Correction of F_v/F_m for the PS1 fluorescence would increase the Φ_{PS2max} obtained from STF close to the Φ_{O2max}. In practice, the underestimation and the overestimation of F_v/F_m due, respectively, to PS1 fluorescence and the non-photochemical quenching removed during a long saturating flash seem to cancel each other: the typical value of F_v/F_m estimated from a long multiple turnover flash (0.83) is close to the expected Φ_{PS2max}.

The use of a multiple-turnover actinic irradiation pulse is also appropriate for the estimation of the operational quantum yield of PS2 electron transport estimated by the fluorescence ratio $\Delta F/F_m'$. Several reports show strong correlations between Φ_{CO2} in plants adapted to different irradiances and $\Delta F/F_m'$ induced by ≈1 s irradiation pulse (Genty *et al.* 1989, Krall and Edwards 1991, Valentini *et al.* 1995). Despite some variations in the literature, the typical relationship between $\Delta F/F_m'$ and Φ_{CO2} is characterized by a linear curve with a slope slightly above the theoretical value of 9.2 ($\Delta F/F_m' = 4\ \Phi_{CO2}/(0.87\times0.5) = 9.2\ \Phi_{CO2}$) (see Valentini *et al.* 1995). Importantly, the linear curve extrapolates at or near the origin at very high irradiances in agreement with the theory (Genty *et al.* 1989, Seaton and Walker 1990).

As for F_v/F_m, the values of $\Delta F/F_m'$ and of the photochemical quenching coefficient q_P are consistently lower when calculated from the F_m' yield induced by a saturating STF compared to those induced by a long irradiation pulse (Schreiber *et al.* 1995b, Prasil *et al.* 1996). However, these lower values are still qualitatively meaningful for the assessment of Φ_{PS2} and q_P as indicated by the close correlations (r^2>0.99) between ΔF/F_m' (Fig.1*A*) and between q_P (Fig. 1*B*) estimated from a long irradiation pulse and from a short STF measured in tomato leaves adapted to different irradiances by red radiation.

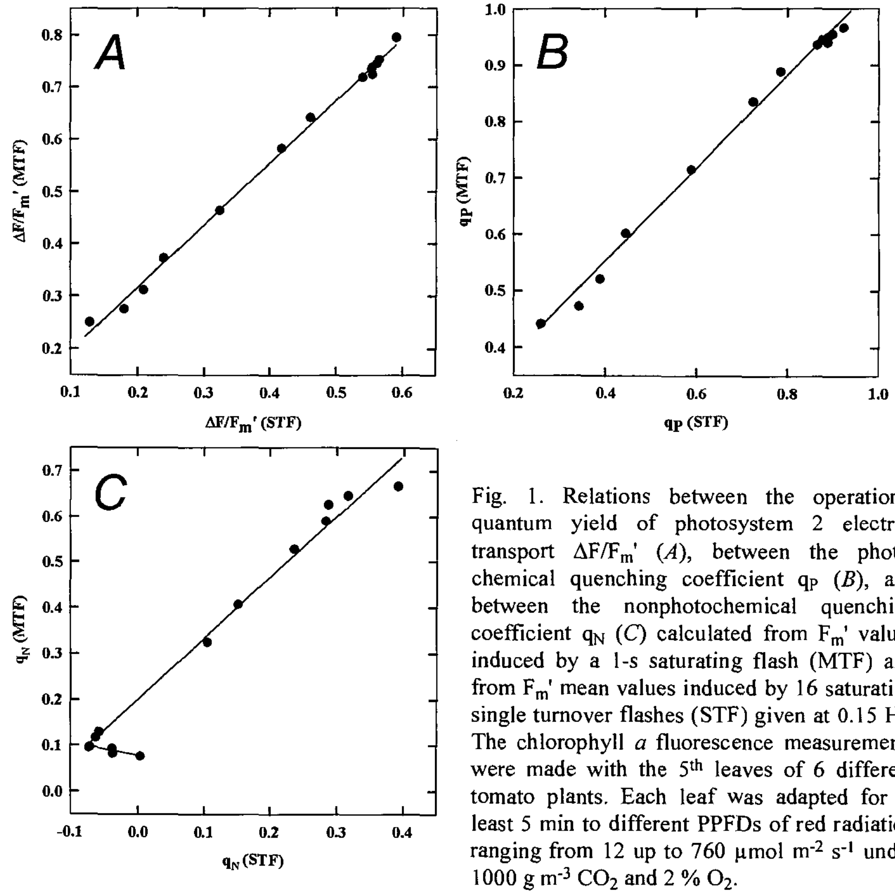

Fig. 1. Relations between the operational quantum yield of photosystem 2 electron transport ΔF/F_m' (*A*), between the photochemical quenching coefficient q_P (*B*), and between the nonphotochemical quenching coefficient q_N (*C*) calculated from F_m' values induced by a 1-s saturating flash (MTF) and from F_m' mean values induced by 16 saturating single turnover flashes (STF) given at 0.15 Hz. The chlorophyll *a* fluorescence measurements were made with the 5th leaves of 6 different tomato plants. Each leaf was adapted for at least 5 min to different PPFDs of red radiation ranging from 12 up to 760 µmol m^{-2} s^{-1} under 1000 g m^{-3} CO_2 and 2 % O_2.

On the contrary, the relationship between q_N estimated from long and short saturating flashes deviates abruptly from linearity at low to moderate photosynthetic photon flux density (PPFD) (Fig. 1*C*). As observed by Schreiber *et al.* (1995b) with spinach leaves adapted to different PPFD, in our experiments with tomato leaves (Fig. 1*C*) and also with potato leaves (Yaakoubd *et al.*, unpublished), the amplitude of the photochemical phase is not quenched and even increases slightly as PPFD increases from 0 to about 200 µmol m^{-2} s^{-1} and then decreases at higher PPFDs. As a result, there is a "negative nonphotochemical quenching" at low to moderate PPFD when q_N is calculated relative to the dark-adapted F_m level (Fig. 1*C*). This biphasic behavior could likely indicate the presence of two concurrent processes as the PPFD increases: first the removal of the nonphotochemical quenching responsible for the thermal phase (possibly related to the increased reduction of the PQ pool), and then the induction of the q_N quenching related to ΔpH and the violaxanthin cycle (Pfündel and Bilger 1994). Above a given PPFD, q_N estimated from a STF and MTF increases in a constant proportion. With a high time resolution, the photochemical phase (J-step) and the nonphotochemical phases (I- and P-steps) can be measured during the fast fluorescence rise and the linear relationship between the two types of phases has been shown for *Scenedesmus* under physiological conditions (Strasser B.J. *et al.* 1999).

Distinct susceptibilities of the photochemical and thermal phases to inhibitors and environmental stresses

In the previous section, we discussed how the distinction between the photochemical and thermal phases of Chl *a* fluorescence can influence the assessment of electron transport efficiency. Here, we focus on the distinct responses of both phases to different inhibitors of PS2 electron transport and to environmental stresses.

As mentioned above, inhibition of electron transport on the PS2 acceptor side by DCMU causes an increase of the photochemical phase of Chl *a* fluorescence at the expense of the thermal phase, so that the F_m yield remains in most cases unchanged (Neubauer and Schreiber 1987, Lazár *et al.* 1998). Similar effects were also observed by other inhibitors acting on the PS2 acceptor side, such as *o*-phenanthroline, ioxynil, and phenylurethan (Delosme 1967, Neubauer and Schreiber 1987). However, it remains to be determined how closely the increase of the photochemical phase (or alternatively, the decrease of the thermal phase) relate to the inhibition of PS2 electron transport.

On the other hand, it was recently demonstrated that the inhibition of the PS2 donor side by Tris- or hydroxylamine-treatments, as well as by an allelopatic compound extracted from the wood of *Cedrela odorata* has practically no quenching effect on the photochemical phase of Chl *a* fluorescence induction whereas the thermal phase is completely abolished (Achnine *et al.* 1998). After a complete inhibition of the PS2 donor side, the maximum fluorescence level is reached about 1 ms after the onset of the strong actinic irradiation and then gradually declines (Strasser B.J. 1997). Such fluorescence transients indicate that the primary photochemistry in PS2 RCs is not impaired by these treatments but cannot be sustained owing to the lack of electron donation from the O_2-evolving complex.

A greater quenching of the slow thermal phase relative to the photochemical phase was also observed after photoinhibitory treatment (Schreiber *et al.* 1995b), in presence of atmospheric pollutants such as SO_2 and NO_2 (Schmidt *et al.* 1990), and after heat treatment (Guissé *et al.* 1995). In the latter case, an additional fluorescence transient called K was detected in the 200-300 μs range using the PEA fluorometer. This rapid fluorescence step was suggested to result from an inhibition of the water splitting system and some changes in the organization of the antennae of PS2 affecting the energy migration properties within PS2 complexes (Srivastava *et al.* 1997).

Conclusions

In the light of recent findings and experimental evidences accumulated over the last three decades, it becomes clear that the basic assumption underlying the fluorescence quenching analysis must be reconsidered in order to take into account the presence of two distinct types of variable Chl *a* fluorescence, *i.e.*, the photochemical and the 'thermal' phases. Indeed, the fluorescence quenching analysis assumes that the saturating irradiation pulse used to induce F_m removes only the photochemical quenching without affecting the degree of nonphotochemical quenching. In dark adapted material, the photochemical and the 'thermal' phases contribute in a similar proportion (50 %) to the total variable Chl *a* fluorescence. They can be distinguished by the use of an STF which induces only the photochemical phase or by a sudden and strong saturating flash during which the rate of Q_A photoreduction exceeds its reoxidation rate: at these high irradiances, the kinetics of the fast photochemical phase is determined by the rate of PS2 primary photochemistry whereas the rise of the slow thermal phase is saturated (Neubauer and Schreiber 1987).

Contrary to the photochemical phase, the nature of the thermal phase is still unclear. At this stage, it can be considered as a nonphotochemical quenching that is removed within few hundreds of ms during a saturating irradiation pulse. This quenching most likely occurs in the PS2 antenna and may be somehow determined by the redox state of the PQ pool and/or the degree of occupancy of the Q_B binding site of PS2 by oxidized PQ molecule. It is independent from q_N associated to the ΔpH and the xanthophyll cycle that is generated during prolonged irradiation (Pfündel and Bilger 1994).

Considering their distinct characteristics, the photochemical and thermal phases constitute an *intrinsic* heterogeneity of variable Chl *a* fluorescence that is not related to the PS2 antenna and PS2 reducing side heterogeneities (Melis 1991). Upon a saturating flash, the induction of variable Chl *a* fluorescence initially reflects the primary photochemical reactions and charge stabilization in PS2 RCs and then it becomes influenced by other reactions occurring within or near the PS2 complexes (Govindjee 1995). Therefore, the two phases of variable Chl *a* fluorescence discussed here do not indicate some structural heterogeneities between different populations of PS2 complexes.

Despite its quantitative importance, the distinction between the photochemical and thermal phases of Chl *a* fluorescence does not affect qualitatively the estimations of the maximum (F_v/F_m) and the operational ($\Delta F/F_m$) quantum yields of PS2 photochemistry (Fig. 1*A*; see also Schreiber *et al.* 1995a,b). In practice, the use of MTF provides closer estimates of the actual quantum yields of PS2 photochemistry that can be deduced from measurements of the quantum yields of O_2 evolution (Björkman and Demmig 1987) or CO_2 assimilation (Long *et al.* 1993). Alternatively, the use of STF would also provide good quantitative estimates of Φ_{PS2} if the contribution of PS1 fluorescence (30 and 50 % of the total F_0 level in C3 and C4 plants, respectively (Genty *et al.* 1990a,b, Pfündel 1998) was taken into account.

On the other hand, the estimations of q_N calculated from F_m induced by either an STF or MTF can largely differ, especially at low to moderate ambient irradiances. The amplitude of the photochemical phase of Chl *a* fluorescence in chloroplasts adapted to different irradiances seems to be determined by two independent processes, *i.e.*, the removal of the nonphotochemical quenching related to the increased reduction state of the PQ pool, and the induction of q_N related to the ΔpH and the xanthophyll cycle. Furthermore, the different responses of the two phases of variable Chl *a* fluorescence toward various inhibitors and environmental stresses can help to provide a closer relationship between Chl *a* fluorescence changes and the inhibition of PS2 electron transport.

As a result of the recent progress in instrumentation development new techniques are available that allow to separate clearly both parts of intrinsic Chl *a* fluorescence heterogeneity. In this way the progress in instrumentation will help to bridge the existing gap between the available technology and our understanding of the Chl *a* fluorescence.

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