



Contribution to the discussion on the nonregulatory nonphotochemical quenching

Revisiting the nonregulatory, constitutive nonphotochemical quenching of the absorbed light energy in oxygenic photosynthetic organisms

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Abstract

The present paper aims to open discussion on the information content, physical mechanism(s), and measuring protocols to determine the partitioning of the absorbed light energy in oxygenic photosynthetic organisms. Revisiting these questions is incited by recent findings discovering that PSII, in addition to its open and closed state, assumes a light-adapted charge-separated state and that chlorophyll *a* fluorescence induction (ChlF), besides the photochemical activity of PSII, reflects the structural dynamics of its reaction center complex. Thus, the photochemical quantum yield of PSII cannot be determined from the conventional ChlF-based protocol. Consequently, the codependent quantity – the quantum yield of the so-called nonregulatory constitutive nonphotochemical quenching (npq) – loses its physical meaning. Processes beyond photochemistry and regulatory npq should be identified and characterized by multifaceted studies, including ChlF. Such investigations may shed light on the putative roles of dissipation and other energy-consuming events in the stress physiology of photosynthetic machinery.

Keywords: chlorophyll *a* fluorescence; constitutive nonregulatory dissipation; F_v/F_m ; nonphotochemical quenching; quantum yield; structural dynamics.

The activity of Photosystem II (PSII) *in vitro* and *in vivo* is routinely monitored by recording chlorophyll *a* (Chl *a*) fluorescence induction (ChlF), which is arguably the most widely used technique in photosynthesis research – it is employed in stress biology and molecular biology of oxygenic photosynthetic organisms, terrestrial and marine ecophysiology as well as in biotechnology of cyanobacteria

and algae, and agriculture of cultivated plants (Strasser *et al.* 1995, Maxwell and Johnson 2000, Roháček 2002, Govindjee and Papageorgiou 2004, Baker 2008, Murchie and Lawson 2013, Kalaji *et al.* 2014, Gorbunov and Falkowski 2022, Mattila *et al.* 2022): these studies, and many more, advanced substantially our knowledge about the light reactions of photosynthesis. The use of ChlF

Highlights

- Chlorophyll *a* fluorescence induction (ChlF) is a key tool in photosynthesis research
- ChlF is not suited to determine the quantum yields of photochemistry and dissipation
- Partitioning of the excitation energy should be studied by multifaceted techniques

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Abbreviations: Chl – chlorophyll; ChlF – chlorophyll *a* fluorescence induction; DCMU – *N*-(3,4-dichlorophenyl)-*N,N*-dimethylurea; F_m / F_m' – maximum Chl *a* fluorescence yield in the dark-adapted / light-adapted state; F_o / F_o' – minimum Chl *a* fluorescence yield in the dark-adapted / light-adapted state; F_v / F_v' – variable Chl *a* fluorescence in the dark-adapted / light-adapted state, $F_v = F_m - F_o$ / $F_v' = F_m' - F_o'$; npq – nonphotochemical quenching of the absorbed light energy; NPQ – regulatory npq $(F_m - F_m')/F_m'$; PSII_{O/C/L} – open/closed/light-adapted state of PSII; RC – photochemical reaction center; STSF – single-turnover saturating flash; $Y_{(II)}$ – quantum efficiency of the photochemistry of PSII; $Y_{(NO)}$ – quantum efficiency of the so-called nonregulatory constitutive npq; $Y_{(NPQ)}$ – quantum efficiency of the regulatory npq; $\Delta\tau_{1/2}$ – half-waiting time between consecutive STSFs inducing ChlF increment.

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in all these diverse areas has been and is being aided by advanced, highly elaborated commercially available instruments (Trtílek *et al.* 1997, Schreiber *et al.* 2012, Kalaji and Goltsev 2020, Lazár *et al.* 2022) and home-built devices (Prášil *et al.* 2018) as well as by theoretical works (Butler and Strasser 1977, Lazár 2015).

The ‘mainstream’ interpretation of ChlF transients in the past decades was based on the so-called Q_A model, according to which the rise from the minimum (F_o) to the maximum (F_m) of ChlF of dark-adapted PSII reflects the closure of all functionally active reaction centers (RCs), which possess two states, the quenched or open state (PSII_o) and the unquenched or closed state (PSII_c), containing oxidized and reduced quencher/ Q_A , respectively (Duysens and Sweers 1963, Stirbet and Govindjee 2012). Here Q_A is the first, stable quinone electron acceptor of PSII. (Most Chl fluorimeters resolve intermediate phases, such as the J and I or the I₁ and I₂ phases, upon rising from the minimum level, O, equivalent to F_o , to the maximum level, P, equal to F_m .)

Important physical quantities have been determined within the framework of the Q_A model. The procedures are based on measured (F_o and F_m) and calculated ($F_v = F_m - F_o$) parameters and kinetic traces of ChlF and on a few explicit and implicit assumptions. These quantities, described in (i)–(iii) below, served as ‘pillars’ until very recently in most studies using ChlF. (i) Assuming first-order de-excitation processes of the singlet-excited Chl *a* via photochemistry, fluorescence, and dissipation, the maximum photochemical quantum yield of PSII, hereafter denoted as $Y_{(II)}$, was equated with F_v/F_m for the dark-adapted state (Kitajima and Butler 1975, Butler 1978). For the derivation of the equation of $Y_{(II)} = F_v/F_m$, and the analogous equation for the light-adapted state, with F_o' , F_m' levels and $F_v' = F_m' - F_o'$, see *e.g.*, Blankenship (2021) and Garab *et al.* (2023). It is interesting to point out that typical *in vivo* and *in vitro* F_v/F_m ratios are found between about 0.7 and 0.8 (Wientjes *et al.* 2013, Santabarbara *et al.* 2019, Sipka *et al.* 2021); in unstressed leaves typically value is 0.83 (Murchie and Lawson 2013). (In this paper I use $Y_{(II)}$ as the photochemical quantum efficiency of PSII in the most general form, not dependent on ChlF parameters.) (ii) The observed sigmoidal rise of ChlF, in the presence of *N*-(3,4-dichlorophenyl)-*N,N*-dimethylurea (DCMU) was assigned to reflect energetic connectivity of PSII RCs (see Stirbet 2013). DCMU inhibits the electron transfer between Q_A and Q_B , the primary and secondary quinone electron acceptor, and thus allows only one stable charge separation in PSII. (iii) Because Y_Σ , the sum of quantum efficiencies, $Y_{(II)}$ (photochemistry) and $Y_{(NPQ)}$ (‘regulatory’ npq, nonphotochemical quenching), if determined using ChlF, was found to be smaller than unity, ‘constitutive, nonregulatory npq component’, $Y_{(NO)}$, was introduced to account for the partition of the absorbed light energy in PSII: $Y_\Sigma = Y_{(II)} + Y_{(NPQ)} + Y_{(NO)} = 1$ (Genty *et al.* 1996, Kramer *et al.* 2004). Note that $Y_{(II)} + Y_{(NPQ)} < 1$ followed almost automatically from the facts that $Y_{(II)}$ was equated with F_v/F_m (≤ 0.8) and that in the dark-adapted sample, NPQ is absent, and thus $Y_{(NPQ)} = 0$. Here, $Y_{(NPQ)} = (F/F_m)(1 - F_m'/F_m)$ (Genty *et al.* 1996) and

$NPQ = (F_m - F_m')/F_m'$ (Murchie and Lawson 2013). For the mechanisms of NPQ, see Horton (2012) and Ruban and Wilson (2021).

The immense success of ChlF has been shadowed by controversies over the Q_A model since the 1960s, which persisted and culminated in the past five years (reviewed in Garab *et al.* 2023, which also accounted for models and technical approaches alternative to the mainstream model and measuring techniques). It became evident that besides photochemical activity, ChlF carries information about the structural dynamics of closed PSII RC complexes, and that illumination of closed RCs leads to the gradual formation of PSII_L, the light-adapted charge-separated state that possesses increased stability of charges compared to PSII_c (Sipka *et al.* 2021). With regard to ChlF parameters, it has been confirmed that in DCMU-treated samples the first STSF, generating PSII_c in all active RCs, induces an F_1 ($< F_m$) fluorescence level, and additional excitations are required to reach F_m (Joliot and Joliot 1979); further, as it was discovered by Magyar *et al.* (2018), to achieve the F_i -to- $F_{(i+1)}$ increments ($i = 1, 2, 3 \dots$), and to reach F_m , sufficiently long $\Delta\tau$ waiting times are required between consecutive STSFs. Increase in the fluorescence yield of PSII_c was also observed in intact leaves of vascular plants by Laisk and Oja (2020), who found that the level of ChlF upon the ‘immediate’ rise, measured at 40 μ s (orders of magnitude longer than required for Q_A reduction), was merely $1.8 \times F_o$. These data and the marked temperature dependence of the $\Delta\tau_{1/2}$ half-waiting times between about 300 and 170 K and its dependence on the lipidic environment of the RCs as well as the strong decrease of the F_1 level at cryogenic temperatures combined with the retained ability of the F_m , but not the F_1 , to relax below 250 K (Magyar *et al.* 2018, 2022, 2023), pointed to the involvement of at least two different physical mechanisms in the dark-to-light transition, *i.e.*, in the F_o -to- F_1 rise and the F_1 -to- F_m multistep increments (Garab *et al.* 2023).

In the following paragraph, I will dismantle one by one the above-identified pillars of the conventional interpretation of ChlF. However, at the same time, I would like to emphasize that doing it opens new perspectives for a deeper understanding of the structural dynamics of PSII and the underlying physical mechanisms in PSII (Sipka *et al.* 2022). Clarifying these questions is also mandatory for the correct analysis of the ‘big data’ of ChlF, which has already been commenced (Kuhlgert *et al.* 2016) and which may soon become part of the everyday practice in plant biology and agriculture (Keller *et al.* 2024).

(i) Concerning the quantum efficiency of PSII, as equated with the F_v/F_m ratio, it is clear that using the F_1 level, reached upon closing all PSII_o, would provide unrealistically low values for $Y_{(II)}$. The explanation, that resolves this discrepancy, is offered by recent steady-state and time-resolved Chl *a* fluorescence spectroscopy data which have shown that the explicit and implicit assumptions used by Kitajima and Butler (1975), listed by Blankenship (2021), cannot be justified (Garab *et al.* 2023). (The assumptions: “none of the rate constants change as the traps go from open to closed, and all the fluorescence observed in both the F_o and F_m states come from

a homogeneous system in which all chlorophyll excited states are equivalent".) It is a delicate question as to how far the F_v/F_m ratio can be used as a 'convenient' proxy of $Y_{(II)}$, by convention and because of well-documented cases of close correlation between photosynthetic efficiency and this parameter (Genty *et al.* 1989). My opinion is that while the F_v/F_m ratio is a useful parameter of PSII to compare wild-type and not-too-distant mutant organisms or samples before and after a not-too-harsh treatment, it should not be used as a proxy of $Y_{(II)}$. For instance, in the case of photoinhibition, RC heterogeneity (active and inactive centers) may more readily explain the decreased F_v/F_m ratio than a decreased (and nonspecified) quantum efficiency (Kono *et al.* 2022). More detailed investigations may reveal different recombination pathways in PSII and the production of 1O_2 which then damages the RC (Vass and Cser 2009, Mattila *et al.* 2023); it is equally important to detect triplet states that are involved in alternative mechanisms of excess excitation dissipation (Laisk *et al.* 2024). In some mutants, the structural dynamics of PSII may remain concealed if, instead of a more detailed investigation, proxy- $Y_{(II)}$ is used. To realize how misleading the use of F_v/F_m ratio is as quantum efficiency, see Fig. 1. (ii) The origin of the sigmoidal rise can easily be explained by invoking more than one exponential rise (Vredenberg

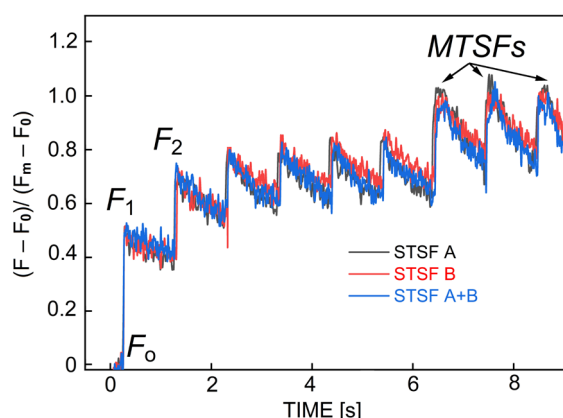


Fig. 1. ChlF transients of isolated DCMU-treated PSII core complexes of *Thermotichus vulcanus*. The transients, recorded at 5°C, were elicited by a series of STSFs, followed by multiple-turnover saturating flashes (MTSFs) as indicated by arrows. Similar traces have earlier been recorded (e.g., Magyar *et al.* 2018, Sipka *et al.* 2019) to convince ourselves that the applied short Xe flashes are indeed saturating and that increasing the intensity of the exciting flashes (by applying the two flashes simultaneously) do not induce fluorescence quenching. Note also that the 'second' STSF, without Δt , is not capable of generating the F_1 -to- F_2 rise. Concerning the quantum yield of PSII, it is clear that no reliable $Y_{(II)}$ value can be derived from these data. The incongruity of using the F_v/F_m ratio (~ 0.77 in this case), or the $F_1/(F_1 - F_0)$ ratio, as a proxy for $Y_{(II)}$ is underlined by the fact that the measured fluorescence levels (at the given time resolution) are insensitive to doubling the excitation intensity. Since no NPQ is detected, with the two simultaneously fired STSFs the magnitude of $Y_{(NO)}$ must be at least 50%, i.e., equal to or larger than that of $Y_{(II)}$. Spectroscopic techniques with high time resolution would most certainly reveal the formation of charge recombination, triplet states, and possibly other processes, as constituents of $Y_{(NO)}$.

2008), which is the case even for isolated dimeric or monomeric core complexes (CCs) of PSII (Magyar *et al.* 2018, Sipka *et al.* 2021). Further, data presented by Oja and Laisk (2020) strongly suggest the absence of excitonic connectivity between PSII units in higher-plant leaves. (iii) Concerning the derived parameter $Y_{(NO)}$, it is clear that it is possible to close all RCs without sizeable excess excitation, and thus the quantum efficiency of PSII can be close to 100%. This can be verified e.g., by absorbance transients of electrochromic shifts and C_{550} (Joliot and Joliot 1979, Sipka *et al.* 2021). Under these conditions, unlike using the F_v/F_m ratio, the magnitude of $Y_{(NO)}$ becomes negligible. In practice, because of charge recombination and miss parameters, the maximum quantum yield of PSII photochemistry is estimated to be about 90% (Shevela *et al.* 2023).

Based on the arguments above, it may be tempting to dismiss $Y_{(NO)}$ as such. However, under most conditions, the sum of $Y_{(II)} + Y_{(NPQ)}$ is substantially lower than unity, and thus, without $Y_{(NO)}$, a large fraction of the absorbed quanta would not be accounted for. This means that the occurrence of npq-silent dissipative processes is more the rule than the exception. Thus, in agreement with Genty *et al.* (1996) and Kramer *et al.* (2004), $Y_{(NO)}$ must be introduced, albeit the original protocol to determine its magnitude cannot be used. Elucidating the nature and physical mechanisms of the processes accounted for by $Y_{(NO)}$ requires multifaceted techniques, which may include absorption transient and Chl *a* fluorescence lifetime measurements as well as steady-state and time-resolved circular dichroism, Raman, and FTIR spectroscopy. It is also worth noting that a fraction of the absorbed quanta might drive other, nondissipative processes, e.g., the formation of reactive oxygen species. Detailed studies on $Y_{(NO)}$ may contribute to a better understanding of the stress physiology of oxygenic photosynthetic organisms.

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