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Contribution to the Discussion on the Nonregulatory Nonphotochemical Quenching

LETTER TO THE EDITOR

Response to the article by Gyözö Garab in *Photosynthetica* (DOI: 10.32615/ps.2024.022) with the title: Revisiting the nonregulatory, constitutive nonphotochemical quenching of the absorbed light energy in oxygenic photosynthetic organisms

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Being personally invited by Gyözö Garab to contribute to the discussion of his recent article in *Photosythetica*, I have chosen the form of this Letter to the Editor to articulate my personal opinion and perspective. My comments relate to both, the aspect of nonregulatory nonphotochemical quenching (emphasized in the title) and the experimental evidence on which the conclusions of Gyözö Garab and his co-workers are based.

Before going into the discussion of details I would like to take a brief look back at the development of PAM fluorimetry and the Saturation Pulse (SP) method, with the help of which about 40 years ago the determination of F_v/F_m and the complementary PSII quantum yields Y(II), Y(NPQ), and Y(NO) became possible (Schreiber *et al.* 1986, Schreiber 2004). Using this approach, for the first time the photosynthetic performance of leaves (and other organisms) in their natural environment could be investigated nonintrusively. Notably, quantitative information (*e.g.*, on photosynthetic capacitance) was obtained most reliably under steady-state conditions of illumination, *i.e.*, after equilibration of all regulatory mechanisms involved in overall photosynthesis. Before the advent of PAM fluorimetry, fluorescence-based information on *in vivo* photosynthesis mainly relied on the phenomenology of dark-light induction (Kautsky effect), the enormous complexity of which sets narrow limits to a reliable interpretation.

The rationale of the SP method is simple: it is based on the fact that the sum of the quantum yields of photochemical energy conversion, fluorescence emission, and heat dissipation is unity. With the application of an SP, the photochemical quantum yield becomes zero, so that the energy that is distributed to PSII ends up in fluorescence and heat. The nice thing about PAM fluorimetry is that the relative fluorescence yield can be directly measured at any point of time (before an SP and during an SP), both in the dark-adapted and in any illuminated state. To derive quantitative information from thus determined relative fluorescence yields, some assumptions have to be made:

(1) The SP does cause complete closure of photochemical energy conversion in PSII.

(2) During the SP there is no change in the ratio between fluorescence and heat formation.

(3) The fluorescence originates in PSII, *i.e.*, it is F(II) and the contribution of F(I) is either negligibly small or corrected for.

(4) PSII is homogenous, *i.e.*, the extent by which various types of PSII heterogeneity (described in the literature) affect the results, is negligible.

There can hardly be any doubt that this approach has proven an exceptionally useful tool in various fields of basic and applied photosynthesis research. This is particularly true for the derived parameters $F_v/F_m = (F_m - F_o)/F_m$ (after dark adaptation) and $F_v'/F_m' = (F_m' - F)/F_m'$ (during illumination) as well as NPQ = $(F_m - F_m')/F_m'$. In the evaluation of the results, however, the caveat of the above-mentioned four assumptions should always be kept in mind.

Assumption (2) is of particular interest in the context of the present discussion on nonphotochemical quenching of F_v. Already in the very first reports on PAM fluorimetry, it was demonstrated that during the course of an SP complex changes in relative fluorescence yield are induced, which are characterized by the four basic levels F_o, I_1, I_2, and F_m (polyphasic rise kinetics). The O-I_1 transient is limited by quantum absorption in PSII, whereas the I_1-I_2 as well as I_2-F_m transients are governed by thermal reactions. The fluorescence yield induced by a saturating single turnover flash (ST) was found to be close to the saturated I_1-level (Schreiber 1986, Neubauer and Schreiber 1987).

Hence, from the very beginning it has been clear that for reaching maximal fluorescence yield F_m, full reduction of the primary acceptor Q_A is a necessary but not sufficient condition. While there has been general consensus that the light-driven closure of PSII reaction centers is responsible for the elimination of photochemical quenching

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during the O-I_1 phase, the mechanisms of the remaining nonphotochemical quenching at the I_1- and I_2-levels until today have been a matter of debate. Evidence was presented that the disappearance of this nonphotochemical quenching on one hand parallels the reduction of the PSII acceptor pool (Neubauer and Schreiber 1987) and on the other hand is counteracted by weakening of the PSII donor side (Schreiber and Neubauer 1987). In particular, the observed period-4 oscillation of the I_1-level as a function of the number of preilluminating flashes has been considered strong evidence for the involvement of "donor-side-dependent quenching" (DQ). From the present point of view, such quenching may be explained by membrane domains that contain PSII in the states $S_2YzP680^+ Q_A^-$ or $S_3YzP680^+ Q_A^-$ (in equilibrium with S_2Yz^+ P680 Q_A^- or S_3Yz^+ P680 Q_A^-). $P680^+$ quenches fluorescence yield about equally well as an open reaction center. In addition, DQ may also be caused by charge recombination in the state $P680^+ Q_A^-$, which in the presence of DCMU is stimulated whenever electron donation by the OEC (S-state advancement) is slowed down.

To my knowledge, all oxygenic photosynthetically active organisms, the polyphasic fluorescence rise kinetics of which have been reported on in the literature, display a distinct I_1-I_2 phase, which means that during the later course of an SP, the nonphotochemical quenching at I_1 is suppressed in parallel with the filling of the PSII acceptor pool. In this case, as F_m and F_m' are determined at the end of the SP, the above-stated assumption (2) is fulfilled and the derivation of quantitative information based on the measured parameters F_o, F_m, F, and F_m' is correct. Therefore, in my opinion, in normal photosynthetically active organisms the expressions derived for the fluorescence-based complementary PSII quantum yields Y(II), Y(NPQ), and Y(NO) are valid (Hendrickson *et al.* 2004, Kramer *et al.* 2004, Klughammer and Schreiber 2008), with the caveat that also the other three assumptions have to be fulfilled. In this context, as to assumption (1) it is clear, that this is not fulfilled when the OEC is damaged and the PSII acceptor pool cannot be filled up. Assumption (3) is problematic because there is always F_o(I) and after dark-adaptation also F_v(I). However, F_o(I) can be estimated and corrected for. As to F_v(I), this recently was shown to correspond to the I_2-F_m transient (Schreiber and Klughammer 2021, Schreiber 2023). Hence, the derived expressions should be based on I_2 instead of F_m. In the illuminated state, when the reactions downstream of PSI are light-activated, F_v(I) does not play any role. There remains the homogeneity aspect in assumption (4), the justification of which is not an easy task and because of its complexity shall not be further discussed.

The conclusions of Gyözö Garab and his coworkers regarding the validity of F_m and F_m' determination by the SP method, are mainly based on measurements using PSII core complex samples of the thermophilic cyanobacterium *Thermostichus vulcanus* (reviewed in Garab *et al.* 2023). When measurements were carried out with more intact samples, like isolated spinach thylakoids, sub-freezing temperatures were used. It is well known that electron donation by the OEC is slowed down upon the lowering of temperature. Therefore, to me the ST-induced fluorescence changes in Gyözö's Fig. 1 appear likely to be caused by charge recombination in $P680^+ Q_A^-$ with a quantum yield of about 0.5. In this context, it may be mentioned that in a dilute suspension of *Chlorella vulgaris* in presence of DCMU close to maximal fluorescence yield (corresponding to the I_2-level in the control) is reached within 1 ms after a single saturating ST. A stepwise increase of fluorescence in a series of saturating ST similar to that in Gyözö's Fig. 1 can be induced by hydroxylamine, which is known to affect electron donation by the OEC (Lavergne and Rappaport 1998).

The properties of the observed stepwise increase of fluorescence have led Gyözö Garab and coworkers to differentiate between two distinct states of closed PSII reaction centers, $PSII_C$ and $PSII_L$ (called "light-adapted charge-separated state"), with the latter being formed from the former *via* an unspecified "conformational change" that is supposed to be driven by light (Sipka *et al.* 2021). The basic idea of two sequential hits for reaching maximal fluorescence yield dates back to a model of Valkunas *et al.* (1991) and the double-flash saturation measurements of France *et al.* (1992). The latter data suggested that the saturation curves depend on the flash width, with longer flashes (in the order of 50 µs) giving a sigmoidal pattern with high F_m/F_o and short flashes (in the order of a couple of µs or shorter) a pattern without sigmoidicity and lower F_m/F_o. However, Hemelrijk and van Gorkom (1992) were not able to reproduce these data and pointed out that France *et al.* (1992) underestimated the rate with which Q_A^- is reoxidized in the presence of DCMU. Furthermore, 5 years later, Valkunas *et al.* (1997) presented new model calculations leading to the conclusion that singlet-triplet annihilation is "a more natural explanation for the observations of France *et al.* (1992) than the two-hit model of Valkunas *et al.* (1991)". Very recently, we reported on an advanced new instrument that combines PAM and flash-kinetics measurements and is ideally suited for measurements of double-flash saturation curves (Fig. 12 in Klughammer *et al.* 2024). Using this instrument in measurements with dilute suspensions of *Chlorella* in the presence of DCMU, almost equal saturation curves are obtained, when the same fluence values are applied in the form of either 3-µs or 60-µs ST. In both cases, distinct sigmoidicity in the low fluence range is observed. These observations questions the "two-hit hypothesis" and strongly supports the notion of sigmoidicity being caused by connectivity between PSII units (Joliot and Joliot 1964). The apparent lack of connectivity reported by Oja and Laisk (2012) may be due to the accumulation of Car-triplet states under the applied experimental conditions (strong ST and MT at very low oxygen concentration). Car-triplets undergo an annihilation reaction with Chl-singlets. When formed in a membrane domain with connected PSII units, they can prevent that the effective PSII absorption cross section increases in parallel with the closure of PSII reaction centers.

In conclusion, I do not think that the experimental data and conclusions presented by Gyözö Garab can

seriously shake the foundations laid during the past 50 years by numerous researchers for the interpretation of F_v *in vivo*. The basic laws governing F_v were formulated making assumptions that in reality may not be completely fulfilled. It should be the aim of ongoing basic research on Chl fluorescence to find out under which conditions this is the case and how potential errors can be minimized or corrected for. Like any other experimental method, also SP quenching analysis has its limits of reliability. This is particularly true for samples, the properties of which largely deviate from *in vivo* systems, *e.g.*, fractionated samples and samples at sub-freezing temperatures. Generalizing observations made with such samples is misleading and bound to confuse. Undoubtedly, the PSII core complexes of *T. vulcanus* are a highly interesting object for basic research on PSII and Chl fluorescence. However, the fluorescence data so far published on this type of sample, are quite limited. Combined PAM and flash-kinetics measurements, as recently reported by Klughammer *et al.* (2024), may allow to unequivocally clarify the nature of quenching that cannot be suppressed by a single ST in Fig. 1 of Gyözö Garab.

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