Contribution to the discussion on the nonregulatory nonphotochemical quenching

LETTER TO THE EDITOR

Response to the Letter of Győző Garab in Photosynthetica (DOI: 10.32615/ps.2024.032), as part of the discussion initiated by the paper of Győző Garab with the title: Revisiting the nonregulatory, constitutive nonphotochemical quenching of the absorbed light energy in oxygenic photosynthetic organisms (DOI: 10.32615/ps.2024.022)

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When Győző Garab invited me to this discussion forum, I thought this platform is a good idea, and I spontaneously agreed to participate. As the discussants are allowed to put forward their arguments and counter-arguments less formally than in a regular paper, I expected a vivid exchange of views, so that all participants and readers will be wiser in the end. For my part, I can say that this has already happened and that I see it more clearly. In particular, I have learned from Győző Garab's Letter that his understanding of the concept of Y(NO) differs fundamentally from my own understanding (outlined in Klughammer and Schreiber 2008) and presumably also from that of many other researchers, like Bernard Genty, David Kramer, and Fred Chow, who together with several other researchers carried out careful studies on the partitioning of excitation energy absorbed in PSII between different photochemical and nonphotochemical pathways. As it appears to me, a large part of the controversy is based on an unfortunate misunderstanding on the side of Győző Garab regarding the information provided by PAM fluorimetry and the Saturation Pulse method. To understand this statement, I strongly recommend investing some time in reading the explanations given by Schreiber (2004). Here I just want to recall some important aspects concerning the determination of Y(NO).

The concept of fluorescence-based complementary PSII quantum yields, and Y(NO) in particular, was quite extensively reviewed and explained step by step in Klughammer and Schreiber (2008). It relies on a set of basic assumptions that in my opinion are fulfilled in experiments with leaves, algae, and cyanobacteria. In practice, the value of Y(NO) = F_0/F_m (after dark adaptation) will be correct only, if F_o and F_m are corrected for the contributions of $F_o(I)$ and $F_v(I)$, respectively. In the illuminated state Y(NO) = F/F_m' and just F has to be corrected for $F_o(I)$, as the contribution of $F_v(I)$ in F_m' may be assumed to be negligible. The most important advantage of PAM fluorimetry and the Saturation Pulse method is that valuable information on photosynthetic activity can be gained in any given state of a photosynthetic active organism by monitoring pulse-modulated fluorescence yield and

applying a saturating multi-turnover pulse of light (SP). The SP serves the purpose of inducing non-intrusively a transient state in which the rate of photochemical energy conversion in PSII is suppressed to zero, whereas the rate of nonphotochemical energy dissipation is unchanged to the state before the SP. While numerous studies have confirmed that the latter condition is fulfilled in the case of *in vivo* measurements using appropriately configured SPs (in terms of intensity and width), it has been very clear from the beginning, that this condition is not fulfilled when a saturating single turnover flash (ST) is applied (*see* Figs. 4 and 5 in Schreiber 1986). As we know now, after almost 40 years of searching for the underlying causes, a composite of donor-side dependent quenching (DQ) and carotenoid triplet quenching (TQ) is responsible for the difference between the maximal fluorescence yield that can be determined by a saturating ST compared to an SP (Klughammer *et al.* 2024). This nonphotochemical quenching disappears within about 1 ms after an ST, which cannot readily be monitored in a control sample (due to simultaneous Q_A^- reoxidation), but can be measured in the presence of DCMU by the pump-probe technique (Schreiber and Krieger 1996). We now can state with some certainty that under *in vivo* conditions, the difference between ST and SP-induced maximal fluorescence yield can be satisfactorily accounted for by these two types of nonphotochemical quenching.

Magyar *et al.* (2018) have rediscovered the apparent discrepancy between the maximal fluorescence yields determined by single and multiple turnover protocols in measurements with PSII CC preparations of the thermophilic cyanobacterium *Thermosynechococcus vulcanus* and a variety of other samples. In a series of follow-up papers Garab and co-workers not only claim that the findings obtained by these measurements "resolve

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the controversies regarding the origin of F_v", but also that they **"provide irrevocable experimental evidence showing that the F_v/F_m parameter cannot be used to determine the efficiency of PSII photochemistry"** (Sipka *et al*. 2021).

As far as the fluorescence data presented in these papers are concerned, the multi-step fluorescence rise induced by a train of saturating ST is of central interest, which is depicted in Fig. 1 of Győző Garab's opening paper of this discussion forum. In DCMU-treated samples, a single saturating ST is found to remove part of F_v only and many STs are required to approach F_m, which eventually is induced by an SP. It is considered of particular relevance, that consecutive ST will induce an increase beyond that induced by the first ST only, when a certain dark interval ("waiting time") between consecutive STs is given, the length of which strongly depends on temperature. This is an interesting feature, indeed, the cause of which certainly deserves further studies.

The problem is, that these observations not only are interpreted to reflect "the stepwise formation of a previously unidentified state of PSII, the *light-adapted charge-separated state*", but also taken as evidence "for the occurrence of the sigmoidal rise of fast Chl *a* fluorescence in the absence of connectivity between PSII units" (Sipka *et al*. 2019). While Garab and co-workers assume that this interpretation is in line with the conclusions of France *et al*. (1992), we recently demonstrated a clear-cut sigmoidal flash-saturation curve using 3-μs ST (*see* Fig. 12 in Klughammer *et al*. 2024), thus questioning the conclusions of France *et al*. (1992). While this aspect was extensively discussed in my first Letter, unfortunately, it was totally ignored by Győző in his response Letter, where he continues denying the existence of "energetic connectivity between PSII units". I personally do *not* consider connectivity all that important for the functioning of overall photosynthesis, the rate of which *in vivo* is controlled by energy-dependent, regulated nonphotochemical quenching, NPQ, which renders connectivity ineffective. Its existence in dark-adapted samples, however, appears very important to me, because it tells us something about the assembly of PSII at the pigment–protein complex level and the consequences arising from dimer/monomer organization, both for the functioning of PSII and for the relationship between fluorescence yield and the state of PSII. Furthermore, its denial is problematic, if linked with the proposal of a speculative "light-adapted charge-separated state" and a general questioning of the validity of SP quenching analysis.

In his response Letter, Győző Garab describes measurements in which "a train of single-turnover saturating flashes (STST), instead of an SP" is applied, in an attempt to "generate F_m". He thinks the concept of Y(NO) needs to be revised because he found: "the kinetic traces of ChlF were invariant on doubling the flash intensity (using two simultaneously fired STSFs)", thus resulting in the same F_m, so that irrespective of using one or two flashes the same Y(NO) was determined, *e.g.*, Y(NO) = 0.23 with Y(NPQ) = 0 and F_v/F_m = 0.77. In this context, however,

it is important to recall that the complementary quantum yields, Y(II) + Y(NPQ) + Y(NO) = 1, relate to a given state of the sample *before SP application* (both in the dark and during illumination). The saturating light applied for assessment of F_m just serves an analytical purpose **and *the quantum yield with which this saturating light induces F_m is of no concern***. In normal applications of SP quenching analysis, it has to be assured that the combination of SP intensity and SP width provides sufficiently strong pulses of light to reduce not only the primary PSII acceptor Q_A but also the secondary PQ-pool before reoxidation of the electron transport chain *via* PSI activity sets in. It is just required that the final F_m or F_m' states do not display more non-radiative energy dissipation than before the saturation pulse. Hence, largely oversaturating SP intensities have to be avoided.

To be honest, I am quite puzzled by the "easy-to-perform experiment without the use of a train of single or double STSFs" proposed by Győző. As far as I can see, this "experiment" already was described in Fig. 4 by Neubauer and Schreiber (1987), where the saturation curves of the various characteristic levels of fluorescence yield were depicted. After reaching a maximal level of F_m = P, this level remained practically unchanged when SP intensity was further increased by more than a factor of 5. This shows that the determination of F_m and F_v/F_m by the SP method is quite robust. The same is true for measurements of the polyphasic rise kinetics in Schansker *et al*. (2011) where "the F_v/F_m parameter did not change in a broad range of the photon flux density of excitation". When this leads Győző to conclude that **"the fluorescence-based values of Y(NO) cannot be correct"**, I am afraid that he may not have fully understood the principle of SP quenching analysis and the concept of Y(NO). Once more: **The complementary quantum yields determined by an SP relate to the state *before* the application of an SP**. In the experiment of Győző's Fig. 1, each flash generates a new state and there is a stepwise decrease of Y(II) with each consecutive flash, accompanied by complementary increases of Y(NO) until it reaches zero.

While Győző states in his Letter: "I do sharply disagree with the criticism regarding the use of PSII CC, …", I would like to emphasize that I am fascinated by the ever-improving quality of PSII CC preparations during the past 30 years and that I am eager to carry out measurements with the current high-quality preparations. I am almost sure that important new insights on the functioning of PSII and the relationship between the state of PSII and fluorescence yield can be obtained by applying our new "flash-machine" (Klughammer *et al*. 2024) for dedicated fluorescence studies using these preparations. Hence, **I am far from criticizing the use of PSII CC in general**. But, I still think that observations made using PSII CC should not be overinterpreted and generalized. While it is certainly a great advantage that measurements can be performed without any interference from PSI and F(I), it should be considered that even in most perfect preparations of PSII CC, the components of PSII lack their natural environment. *In vivo*, PSII is embedded in the thylakoid membrane with all kinds of interactions

with other components of the photosynthetic apparatus. Hence, it would be surprising, if all properties of PSII CC would be equal to those of PSII *in vivo*. An important aim of future fluorescence measurements on PSII CC should be to find out which "cofactors" have to be added to render the fluorescence properties of PSII CC more equal to those *in vivo*.

This brings me back to Fig. 1 in Győző's opening paper, where the typical multi-step fluorescence increase to F_m induced by a train of saturating single turnover flashes in the presence of DCMU is shown. As already mentioned in my first response Letter, close to maximal fluorescence yield (corresponding to the I_2 level in the control) can be induced by one single turnover saturating flash using a dilute suspension of *Chlorella* in the presence of DCMU. A stepwise increase is observed, however, in the presence of a low concentration of hydroxylamine, which acts at the PSII donor side (Bouges 1971). But, as "seeing is believing", I have asked the editor the permission to present in this Letter a supplementary figure showing the result of a corresponding experiment carried out with our most advanced new fluorimeter (Klughammer *et al.* 2024). Saturating 3-µs flashes were applied with 5-s dark intervals between consecutive flashes. Flash-induced fluorescence yields were determined at 700 µs and 255 µs after each flash in the presence of DCMU and of DCMU + hydroxylamine, respectively, *i.e.*, after relaxation of carotenoid triplet quenching (TQ) and donor-side dependent quenching (DQ) and before appreciable Q_A reoxidation by the back reaction could take place (for flash-saturation and details on the measuring technique, *see* Fig. 12 in Klughammer *et al.* 2024). F_o-normalized relative PSII fluorescence yield, after correction for $F_0(I) = 35\%$ of overall F_0 (Pfündel 1998, Schreiber and Klughammer 2021), reached a value of about 4.6 under both conditions. With 10 µM DCMU alone, F_m was reached after the first flash, whereas with 10 µM DCMU plus 100 µM hydroxylamine, it was reached in a stepwise manner in a flash train (*see* Fig 1S, *supplement*).

A value of $F_m(II)/F_0(II) = 4.6$ corresponds to $F_v/F_m(II) = 0.78$. This happens to be very close to the F_v/F_m reported by Győző Garab for the dark state of his PSII CC sample (*i.e.*, before application of the first flash) in his Fig. 1. These are the experimental facts obtained with an *in vivo* sample using our most advanced Chl fluorimeter (described in Klughammer *et al.* 2024). Further discussion and research should be focused on the question, of how these facts can be reconciled with what Garab and coworkers have been measuring with their PSII CC and various other samples using the *PAM-101* fluorimeter. I want to emphasize once more that I do not question the quality of the samples. I just want to make clear that **in an intact sample under physiological conditions the "multi-step phenomenology" is observed only after "modification" of the donor side**. This does not contradict the fact that Jian-Ren Shen's PSII CC preparations are excellent, displaying very high rates of oxygen evolution. As already shown by Bernadette Bouges more than 50 years ago, low concentrations of hydroxylamine affect the properties of dark-adapted PSII during the beginning of illumination only, *i.e.*, **the steady-state rate of oxygen evolution is not affected** (Bouges 1971).

Finally, I want to mention that the "waiting-time" phenomenon observed by Garab and coworkers can be reproduced by double-flash measurements with *Chlorella* (Schreiber, unpublished) when 100 µM hydroxylamine is added on top of 10 µM DCMU. This analogy should help identify the true cause of the type of quenching responsible for the multi-step increase of fluorescence yield in the samples used by Garab and coworkers.

In conclusion, I think that the data presented in Győző's Fig. 1 (opening paper) are not at all in conflict with the concept of SP quenching analysis. Therefore, I think that the issue concerning the meaning and correctness of F_v/F_m, Y(II), Y(NO), *etc.*, should be well separated from the question of why in the DCMU-treated samples used by Garab and coworkers many saturating ST are required for reaching F_m. In my opinion, while answering this question is an important task for future research, it should not be linked with the question of whether the data obtained by SP quenching analysis can be trusted. By suggesting such a link, Garab and coworkers have been causing some confusion, which hopefully can be cleared up a little bit by my contributions to this discussion forum. If in our further discussion and research, we concentrate on the differences between highly purified PSII CC and *in vivo* samples, this may lead to some important new insights both on the functioning of PSII and on the relationship between the state of PSII and chlorophyll fluorescence.

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