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Paradoxes in judging the inhibition of photosynthetic electron transfer chain using P700 oxidation and dark re-reduction analyses

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Photosynthesis is the principal natural resource for biological carbon fixation. The Chlorophyll (Chl)-a fluorescence and the gas exchange analyses are the key methods among numerous tools for studying photosynthetic rates in plants. Additionally, during the past 20 years, the photosystem (PS) I redox kinetics analysis has become an important technique to characterize the components of photosynthesis regulation or performance of the photosynthetic electron transfer chain (PETC) between PSII and PSI. For example, interpretation of various phases of PSI redox kinetics have been utilized to draw conclusions from the photosynthetic control on cytochrome *b6f* [1], the role of plastoquinone pool redox state in regulation of linear electron transfer [2] and inhibition of electron flow by various mechanisms [2] as well as on the *in-vivo* gross carbon assimilation [4]. Analysis of different components of PSI redox kinetics has led to conclusions on the functional mechanisms of different proteins involved in photosynthesis regulation, and the redox states of the stromal acceptor pool, by analysis of respective mutant plants [3,5–8]. Applying a saturating pulse method over far-red (FR) oxidized P700 distinguishes acceptor-side limited closed PSI reaction centers (RCs) in addition to the donor-side limited closed RCs and the open centers (true Pm) during the assessment of energy conversion efficiency of PSI in Dual-PAM [9].

Recently, we used the P700 redox kinetics analysis to accurately identify the functions of various intersystem PETC inhibitors, and further validated by gas exchange analysis that the inhibition had occurred, taking advantage from membrane inlet mass spectrometry [10,11]. In these experiments, a simple P700 redox kinetics analysis led to a discovery of malfunctioning of a commonly used photosynthetic inhibitor DNP-INT when applied to isolated thylakoids and leaves [10]. Since this PETC inhibitor has been used in numerous subfields of photosynthesis research [12–15], and our results were recently challenged in a BBA paper [16], we found it important to up-date the photosynthesis community and carefully reiterate the paradox function of DNP-INT.

The different elements and phases of the P700 redox curve, using FR background illumination, are shown in Figure 1, and should be considered when drawing firm conclusions about various processes and functions of the PETC. A choice of examining “wrong phases” of P700 redox curve or P700 difference absorption measurements may lead to floppy conclusions and wrong interpretation of the electron transfer processes. In Figure 1, we demonstrate the redox behavior of P700 in rapidly thawed (-80°C stored), dark incubated thylakoid membranes re-suspended in measuring buffer (described in [10]) before performing the P700 redox kinetics analysis in Dual-Pam-100 liquid sample measuring cavity. The samples analyzed are color-coded. The first part of the figure, comprising a short saturating pulse (SP) of actinic light, given under dark background, demonstrates the maximum redox turnover capacity of P700 in the

control (black trace) and DNP-INT-treated (final concentrations 1 µM (red trace), 10 µM (blue trace), 25 µM (magenta trace) and 100 µM (green trace) thylakoids as well as in DCMU (10 µM) and Methyl Viologen (MV) (10 µM) -treated thylakoids. In the second part of the figure, FR light background illumination was turned on, which preferentially oxidizes P700, except in the acceptor-side limited closed PSI RCs [9]. Subsequent application of a single turnover flash of actinic light (ST, 50 µs) and the following multiple turnover flash (MT, 50 ms) activate the whole chain electron transfer from PSII. The activation of PSII electron transfer by actinic light flashes caused fast re-reduction of P700 by ST and MT flashes, seen as a dip in the curves at 62 and 82 s, respectively. Nevertheless, thereafter, P700 slowly re-oxidized due to continuous FR background illumination.

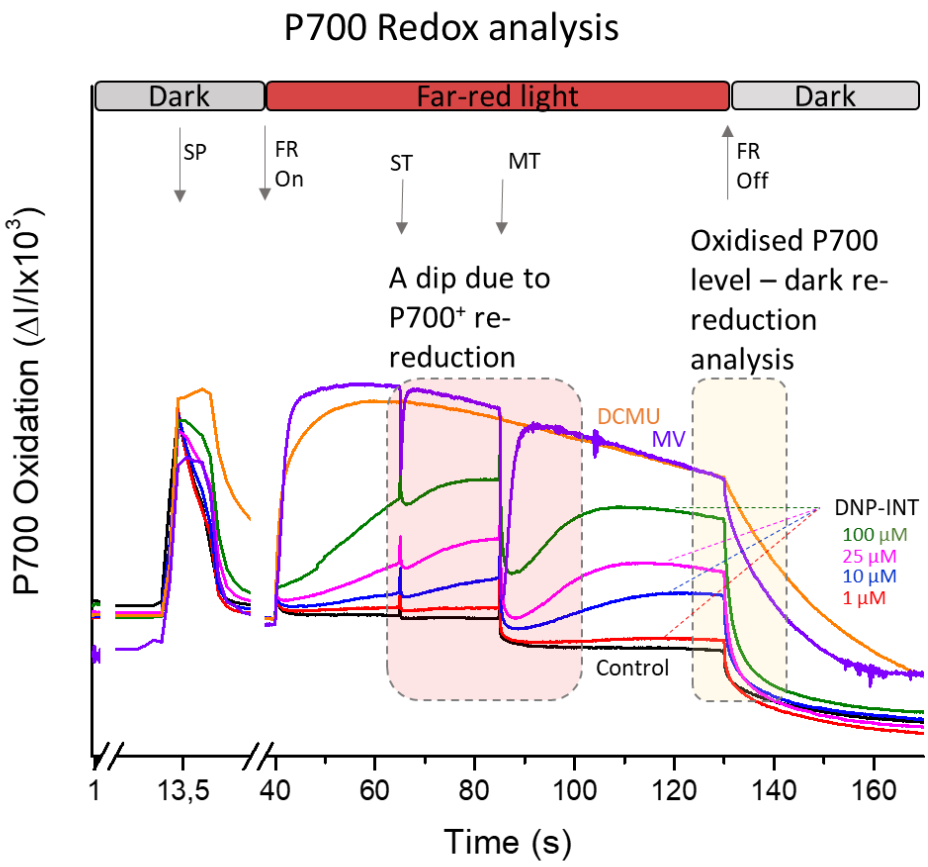

Figure 1. P700 redox kinetics were measured in dark-adapted isolated thylakoid membranes by single turnover (ST, 50 µs) and multiple turnover (MT, 50 ms) actinic light flashes (10000 µmol photons m^{-2} s^{-1}) under a continuous far-red background light (115 µmol photons m^{-2} s^{-1}). Isolated thylakoids equivalent to chlorophyll concentration of 100 µg chl ml^{-1} were suspended in measuring buffer containing 330 mM Sorbitol, 5 mM $MgCl_2$, 10 mM NaCl, 5 mM NH_4Cl, 50 mM HEPES (pH 7.6) for Control or with addition of either Methyl Viologen (MV) (10 µM), DCMU (10 µM) or DNP-INT (1, 10, 25 and 100 µM), each separately.

The pink rectangle in (Fig. 1) highlights the re-reduction of P700 (dips in the curve induced by the ST and MT actinic light flashes) occurring by electron transfer from PSII to a fraction of oxidized $P700^+$, in the untreated control (Black line) and in DNP-INT treated thylakoids. The DNP-INT (in µM) to Chlorophyll (in µg) ratios in these samples were 0.01, 0.1, 0.25 and 1.0, the maximum ratio 1.0 clearly exceeding that (0.8) used in [16]. Any blockage of electron transfer chain between PSII and PSI by DNP-INT inhibition should lead to cessation of re-reduction of P700 by ST or MT actinic light flashes, similar to that demonstrated by the DCMU treated thylakoid sample (orange trace, Fig. 1). The presence of a PSI electron acceptor MV logically allowed faster re-oxidation of P700 after the ST and MT flashes (MV, violet trace) (Fig. 1). Paradoxically, in thylakoid samples treated with increasing DNP-INT concentrations, a gradually enhanced FR-induced P700 oxidation took place after the AL flashes, suggesting an unknown DNP-INT-dependent sink for electrons from PSI. However, with increasing concentrations of DNP-INT the ST and MT flashes induced a broader dip, which indicated a higher leakage of electrons at Cyt *b6f* to plastocyanine, causing reduction of a bigger fraction of oxidized $P700^+$. Indeed, these experiments clearly demonstrate a failure of DNP-INT in blocking the electron transfer from PSII to PSI, in line with our previous work [10]. In the same work, we also applied a semisolid paste of thylakoid membranes on a filter paper for measurements of P700 oxidation in a Dual-Klas-Nir device (shown as supplementary material in [10]). Those samples behaved differently and displayed a shallowing of the dip upon increasing DNP-INT concentrations, which most likely resulted from a limitation of electron transfer activity in a relatively dry smear of thylakoid paste as compared to the fully active thylakoids measured here in a liquid cell.

Finally, in the last part of Figure 1, upon turning off the FR light (at 130s), the whole fraction of closed RCs (oxidized $P700^+$) in all thylakoid samples turned into open RCs (reduced 700) slowly, yet with different rates, as described inside the second yellow rectangular. Noteworthy, this dark-interval relaxation kinetic analysis (DIRK) is often used as a measure of cyclic electron flow [4]. However, in the work [16] the higher level of initial oxidized $P700^+$ and the following dark reduction were misinterpreted as a blockage of electron transfer from PSII to PSI by DNP-INT, completely against the theory of P700 redox kinetics [9]. By detailed demonstration of P700 redox kinetics in the presence of DNP-INT, and a more efficient inhibitor DCMU, we reiterate the paradoxical function of DNP-INT as an inhibitor of intersystem electron transfer and validate our previous finding that DNP-INT is unable to block the electron flow between PSII and PSI. Use of DNP-INT in isolated thylakoids or detached leaves, with an intention to study alternate electron flow to Cytochrome *b6f* or to the PQ-pool, should also be taken with serious precautions.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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