Project Closing Report

The nature and physiological significance of the structural changes of Photosystem II reaction center

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The aim of this report is to summarize our most important findings on the nature and physiological significance of the structural changes of Photosystem II. Studies related to the newly identified light-adapted charge-separated state and waiting-time dependent rate-limiting step(s) will be outlined.

Related publications: *Magyar et al. 2018 Scientific Reports; Sipka et al. 2019 Physiologia Plantarum; Sipka et al. 2021 Plant Cell; Magyar et al. 2022 Photosynthetica*

1. Background

Photosystem II (PSII), which uses solar energy to oxidize water and supplies the reducing equivalents necessary to fix carbon dioxide, is the engine of life. PSII is a large multi-subunit homodimeric protein complex embedded in the thylakoid membranes of cyanobacteria, algae and vascular plants. Its reaction center complex (RC) is surrounded by CP47 and CP43 core antenna proteins. This PSII core complex (PSII CC) may be regarded as the minimal fully functional PSII complex capable of charge separation and stabilization, plastoquinone reduction and oxygen evolution.

Each PSII CC contains the RC incorporated in the D1/D2 proteins, the α and β subunits of cytochrome b_{559} and two integral antenna proteins, CP43 and CP47, which carry 14 and 17 chlorophyll-*a* molecules (Chl-*a*), respectively. The RC D1/D2 proteins are located approximately symmetrically with respect to the transmembrane region. Four Chls (two accessory chlorophylls and a special pair of Chl-*a* molecules, conventionally denoted as P_{680}), two pheophytins, and two plastoquinones (Q_A and Q_B) are arranged in two symmetrical branches.

In the open-state PSII ($PSII_O$), upon the absorption of one photon, electron transfer starts with the formation of the primary radical pair $P_{680}^+Pheo^-$, which is stabilized via the re-oxidation of $Pheo^-$ by the first quinone acceptor molecule Q_A, leading to a charge-separated state $P_{680}^+Q_A^-$. In consecutive steps, P_{680}^+ is re-reduced by the redox-active tyrosine (Y_Z), which then oxidizes the Mn_4CaO_5 cluster, producing the S_2 state of the oxygen-evolving complex (OEC). This generates a closed-state of PSII ($PSII_C$) with all Q_A reduced, which state persists for several hundred microseconds, until the electron is transferred from Q_A^- to Q_B, the secondary quinone acceptor.

Recent time-resolved serial femtosecond crystallography experiments on PSII crystals uncovered structural changes accompanying the reactions around the Q_B/non-heme iron and the Mn_4CaO_5 cluster. This raised the question if the variable chlorophyll fluorescence (F_v) contains any component originating from similar conformational changes.

2. Chlorophyll-*a* fluorescence

Monitoring of PSII activity in vivo or in vitro, upon dark-to-light transition, is routinely performed by recording the rise of fluorescence intensity from the minimum (F_o) to the

maximum (F_m) levels. The variable Chl-*a* fluorescence (F_v=F_m−F_o) follows a complex induction kinetics and carries information on the functioning of the photosynthetic machinery. This is arguably one of the most commonly used techniques in plant biology. However, the origin of Chl-*a* fluorescence transients was still controversial. According to the mainstream concept (Duysens & Sweers 1963, Stirbet and Govindjee 2012), the multiphasic rise from F_o to F_m reflects exclusively the reduction of Q_A. In the so-called OJIP curve (fast fluorescence rise) the letters J and I signify intermediate states, O and P correspond to the F_o and F_m levels, respectively, with F_o ascribed to all Q_A oxidized and F_m to all Q_A reduced. Analysis of OJIP transients in a wide temperature-range revealed (an) additional process(es) of different physical origin in the fluorescence rise once Q_A is reduced (Schansker et al. 2011) and the involvement of light-induced conformational changes in F_v was suggested.

A basic problem with the mainstream model is that in the presence of the Q_A-to-Q_B electron transfer inhibitor DCMU, upon excitation by a single-turnover saturating flash (STSF) only an intermediary level (F_1) is induced and F_m can only be reached by further excitations. It was shown that while the F_o-to-F_1 rise implies the reduction of Q_A, the F_1-to-F_m gradual rise is associated with non-electrogenic reactions – showing their different physical origin. It was hypothesized that these differences are due to the operation a second quencher molecule, Q_2 (Joliot and Joliot 1979). However, the existence of Q_2 has recently been ruled out (Magyar et al. 2018, Sipka et al. 2021).

The observation that the kinetics of the fast fluorescence rise depend on the length rather than on the intensity of the excitation flashes led us to the discovery of the waiting-time related rate-limiting steps in PSII photoreactions. In the presence of DCMU the fluorescence induction displays a sigmoidal rise instead of the expected exponential rise which phenomena was interpreted by the authors within the frameworks of the theory of connectivity between PSII units (Stirbet 2013). On the other hand, it has been also proposed that sigmoidicity might arise from overlapping exponential kinetic components indicating that the long flashes induce a second process raising the fluorescence yield (increases the F_m/F_o ratio) and, because of the kinetic overlap with the first process (the reduction of Q_A), the rise becomes sigmoidal (Vredenberg 2015).

Our investigations on the phenomena mentioned above shed light on the occurrence of conformational changes associated with F_v and uncovered the existence of waiting times between the STSFs, hence of rate-limiting steps which are not the results of either rapid photochemical reactions or heterogeneity of PSII (Magyar et al. 2018). As the excitations leading to the gradual formation of the F_1-to-F_m rise evidently act on $PSII_C$, we propose the manifestation of the stepwise formation of the previously unidentified light-adapted charge-separated state of PSII ($PSII_L$).

3. The light-adapted charge-separated state of PSII

We performed STSF-induced Chl-*a* fluorescence measurements on PSII CCs isolated from *Thermosynechococcus (T.) vulcanus* cyanobacteria and plant thylakoid membranes (TMs) in the presence of DCMU. Our aim was to discriminate between the F_o-to-F_1 ($PSII_C$) and the F_1-to-F_m ($PSII_L$) rises and to clarify their origins. We investigated the differences at cryogenic temperatures where the F_1 levels remain constant, in contrast the F_1-to-F_m increments exhibit strong, temperature-dependent decay characteristics. The amplitude of F_1 and number of excitations required to reach F_m depended strongly on the temperature. This agrees well with that of Joliot and Joliot (1979), who have shown that the first STSF induces an intense electric field but the consecutive steps are not electrogenic. Hence, it was concluded that the first flash reduces Q_A, and it must be followed by several additional excitations to reach F_m.

We interpreted these increments in terms of light-induced conformational changes – which, as shown by us (see below), possess activation energy values and are coupled to the dielectric relaxation in the RC matrix in response to the stationary and transient electric fields around Q_A^- and the $P_{680}^+Pheo^-$ radical pair, respectively. As an alternative, non-conflicting explanation is that dissipation-induced heat packages, which transiently 'melt' the RC matrix, might facilitate the dielectric adjustments.

In order to determine the activation energies of the F_1-to-F_m increment, we analysed the temperature dependence of the F_m decay in PSII CC (ruling out the involvement of PSI) and TMs. The calculated activation energies were 13.8 ± 0.8 kJ/mol and 11.3 ± 3.6 kJ/mol, for TMs and PSII core, respectively, which are in good agreement with each other, and the values derived from the temperature dependences of the OJIP fluorescence rise components in TMs. We also confirmed that the F_1-to-F_m increment originates solely from PSII.

We also investigated the possible effect of the intradimer connectivity of PSII units by comparing the Chl-*a* fluorescence induction of DCMU-treated dimeric and monomeric PSII CCs. The OJIP rises were virtually indistinguishable and both samples displayed a gradual fluorescence rise elicited by a train of STSFs, indicating the absence of significant cooperativity between two monomers.

By using dithionite, and thus pre-reducing Q_A, we confirmed that it is possible to increase the Chl-*a* fluorescence yield in the presence of reduced Q_A ($PSII_C$), gradually reaching F_m ($PSII_L$). On the other hand, high concentrations of dithionite – which pre-reduce Pheo, preventing the formation of $P_{680}^+Pheo^-$ – could terminate these increments showing that the generation of this radical pair is necessary to induce the fluorescence increments in $PSII_C$ and thus to allow the formation of $PSII_L$. C550 absorption measurements – which is diagnostic of the presence of Q_A^- – verified the pre-reduction of Q_A, while thermoluminescence measurements proved that the occurrence of the S_2 state of the OEC was also prevented.

In order to rule out the possible involvement of alternative reductive electron-transfer pathways – like secondary electron transfer from Cyt b_{559} to P_{680}^+ and to tyrosine-D or the S_2 state of the OEC at cryogenic temperatures – we performed STSF-induced Chl-*a* fluorescence and absorption transient measurements by the addition of ferricyanide (FeCy). FeCy showed only little effect on F_v and the stepwise fluorescence rise showed similar kinetics to the DCMU treated control. Absorption measurements at room temperature (RT) showed that electron donation from Cyt b_{559} occurs only in 5-10% of the RCs, probably due to partial impairment of the donor side in some PSII CCs. By measuring the absorbance transients C550 (in PSII CC) and ΔA_{515} (in thylakoids), we confirmed that the second STSF produced no additional stable charge separation even with sufficiently long waiting times between the flashes.

In order to test if the STSF-induced fluorescence increments were caused by the formation and rapid recombination of the $P_{680}^+Pheo^-$ primary radical pair, we performed transient absorption measurements at 819 nm on DCMU-treated PSII CC, using variable waiting times between consecutive laser flashes. Analysis of the decay kinetics of P_{680}^+ confirmed that the kinetics after the first flash could be assigned to the formation of $P_{680}^+Q_A^-$ and re-reduction of P_{680}^+ by the donor side of PSII. In contrast, after the second flash ΔA_{819} decayed much faster, indicating the charge recombination of $P_{680}^+Pheo^-$. This confirms that, in the presence of DCMU, stable charge separation can only be induced by the first saturating flash. We also concluded that the rate-limitation observed in fluorescence experiments does not originate mainly from gating of the primary charge separation.

Light-induced FTIR difference spectra were also recorded after exposing the sample to 1 or 20 STSFs and prominent changes in the 1,800–1,200 cm^{-1} region were observed. The double-difference spectra of the transients revealed evident changes in the amide I region, which is indicative of conformational changes in the protein. These data provide direct

experimental evidence for the involvement of conformational changes associated with the F_1–to–F_m increment. Analysis of the relaxation kinetics of the FTIR signals also revealed differences at physiological temperature. The decay of both the 1,401 cm^{-1} signal originating from the S_2 state of the OEC, and the positive band 1,478 cm^{-1}, characteristic of Q_A^- were slowed down by about a factor of three in $PSII_L$ compared to $PSII_C$.

The newly identified state, the light-adapted charge-separated state, $PSII_L$, is characterized by two quite peculiar features: (1) The $PSII_C$–$PSII_L$ transition can be generated only in a stepwise manner by multiple excitations following the closure of PSII. During this transition no additional stable charge separation is occurring and is driven by a series of rapidly recombining $P_{680}^+Pheo^-$ radical pairs (Magyar et al., 2018; Sipka et al., 2019). (2) When a radical pair is generated "too early" it remains ineffectual, *i.e.* sufficiently long waiting times ($\Delta\tau$) are required between the excitations promoting the transition, in a temperature-dependent manner (Magyar et al., 2018; Sipka et al., 2019).

Another important characteristic of $PSII_L$ – beside the higher fluorescence compared to $PSII_C$, and thus increased fluorescence decay lifetimes and fluorescence yield – is the distinct 80 K emission spectra. This indicates a change in the energy landscape of excitations' trapping/detrapping in the core antenna, which point to reorganizations in the protein matrix in the vicinity of the RC, which is also supported by the FTIR data.

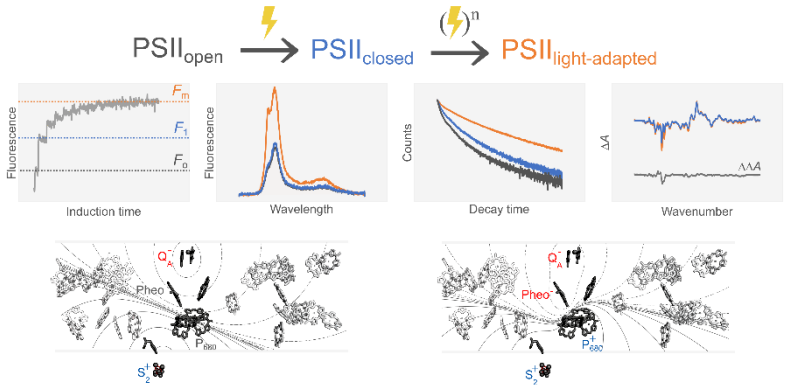

Figure 1. Demonstration of measurements of the $PSII_O$-$PSII_C$-$PSII_L$ transitions in PSII CC, as exhibited by STSF-induced Chl-*a* fluorescence induction, 80 K fluorescence emission, fluorescence lifetimes and rapid-scan FTIR difference spectra. The configurations of the stationary and transient electric fields, due to the stable and transient charge separations are also indicated.

4. The waiting-time related rate-limitation and the effect of the lipidic environment

Our double-STSF induced fluorescence kinetic measurements revealed the involvement of the waiting time ($\Delta\tau$) in all samples tested: TRIS-washed TMs (ruling out the involvement of the Mn_4CaO_5 cluster), PSII core particles (excluding PSII connectivity), intact cyanobacterial cells (PAL mutant of *Synechocystis* PCC 6803, to rule out isolation artefacts). This rate-limiting step was present not only after the first flash but also between later steps – suggesting similar processes during the train of additional STSFs.

With respect to its origin, originally, two possible explanations were hypothesized: (1) It might originate from a rate limitation at the level of primary photochemistry, however as it was shown by transient absorption measurements (Sipka et al. 2019) it does not mean gating of the primary charge separation events, i.e. the generation of the $P_{680}^+Pheo^-$ radical pair. (2) Alternatively, the fluorescence of PSII CC has been proposed to originate from the CP43/CP47 antenna-proteins, and to depend on the excitation energy transfer reactions between P_{680}^* and the domains of the antennas (Shibata et al. 2013). It is reasonable to assume that these processes depend on the physicochemical environment of the RC (hydration state of the sample, conformational mobility of different protein residues, lipid content and composition, *etc.*)

Thus, we investigated the possible effects of the lipidic environment on the origin of the rate-limiting steps in PSII (Magyar et al. 2022). We found that the $\Delta\tau_{1/2}$ values were considerably shorter in TMs and TM liposomes than those in detergent or non-TM liposomes (PC, PC-PE mixture). We found no significant difference in the $\Delta\tau_{1/2}$ half-waiting times between the samples containing additional or only residual amounts of detergent. We emphasized that the waiting time is essentially insensitive to the detergent and lipid shells surrounding the complexes, which is in good agreement with the proposed origin of $\Delta\tau$ from dielectric relaxation processes following variations in the local electric fields inside the RC (Sipka et al. 2021). It still needs to be determined if the role of lipids is in accelerating the dielectric relaxation processes or they facilitate the transduction of the heat packages, associated with the charge recombination events.

In order to see if the lipidic environment has any influence on the excitonic interactions of pigment–protein complexes, we compared the absorption and CD spectra of PSII CCs in solution and reconstituted into native and non-native membranes. We could not observe any sizeable differences between the samples indicating that the lipid/detergent environment does not exert any significant change in the Chl excitonic energies and interactions.

Fluorescence emission spectra were also recorded on PSII CCs in solution and liposomes. The measurements at RT showed no differences, however, the 77 K fluorescence emission spectrum indicated a change in the peak ratios characteristic to red-shifted Chl in CP47 and the one of bulk antenna Chls. The values of the peak ratios of the reconstituted PSII CC membranes were comparable to those in native TMs and whole cells of *T. vulcanus*. This difference in the ratios can be caused by several things, since at 77 K it depends on the dynamic balance of excitation energy trapping by the red-shifted states in CP47 vs trapping in the RC: (1) One explanation can be that at cryogenic temperatures only a partial equilibration occurs with the low-energy Chls at CP47, thus the RC can trap excitations more effectively from CP43. (2) Another explanation is that there might be a partial energetic detachment of CP43 because of the lipids which affects the energetic landscape.

Time-resolved fluorescence spectroscopy with picosecond resolution was also performed to test the possible differences in the excitation dynamics. RT measurements showed rapid decay of PSII CCs with open RCs compared to closed ones with slight differences, thus we applied global lifetime analysis to understand the underlying processes. When the complexes were reconstituted into lipid membranes the average lifetime increased, indicating a slight effect of the lipid environment on the kinetics of excitation energy transfer to the RC. 77 K measurements in F_m ($PSII_L$) state showed considerable changes in the peak ratios and lifetimes when PSII CC embedded in TM lipids. The change of two fastest decay components – indicating the primary charge separation and Q_A^- – implies that the trapping of excitations from CP47 might be faster in TM lipids.

It must be stressed, however, that the changes shown by the fluorescence emission spectroscopy and time-resolved fluorescence spectroscopy are evidently not related to the lipid-dependent variations of the $\Delta\tau_{1/2}$ since the changes were not specific to TM lipids.

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