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The role of protonation processes in the gradual formation of the light-adapted charge-separated state of photosystem II

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Earlier, we discovered that to reach the maximum chlorophyll-a (Chl-a) fluorescence level of photosystem II (PSII) in diuron-treated samples, and thus PSII_L, the light-adapted charge-separated state, multiple excitations with sufficiently long $\Delta\tau$ waiting times between excitations are required. Experiments of Chl-a fluorescence, elicited by single-turnover saturating flashes (STSFs), revealed that the $\Delta\tau_{1/2}$ half-waiting times between flashes depended on the lipid content of the reaction centre (RC) and on the temperature. We hypothesized that the gradual formation of PSII_L depends on protonatable residues (and bound water molecules) at the donor side of the RC. Here, we tested this hypothesis: we used diuron-treated isolated PSII core complexes of *Thermotrichus vulcanus* and determined the pH dependence of $\Delta\tau_{1/2}$ and other Chl-a fluorescence parameters at different temperatures. Data recorded at 5°C revealed moderate and steep increases of $\Delta\tau_{1/2}$ between pH 5.0 and 6.5, and 6.5 and 8.0, respectively. Much weaker pH dependences were seen at cryogenic temperatures (−20 and −60°C), indicating the role of structural plasticity in the protonation-dependent reorganizations. Similar to the strong pH dependence of $\Delta\tau_{1/2}$ at 5°C, the magnitude of the F_1 fluorescence level, induced by the first STSF, displayed moderate and steep increases between pH 5.0 and 6.5, and 6.5 and 8.0, respectively, at all temperatures. Corroborating data were received from fast Chl-a fluorescence transient measurements. Hence, our data provide experimental support to the hypothesized role of protonation processes in the formation of PSII_L. The protonophores, CCCP and FCCP significantly increased the $\Delta\tau_{1/2}$ at pH 6.0 and essentially eliminated the pH dependence of the F_1 fluorescence level. These data provide experimental support to our hypotheses on the role of protonatable residues in determining the structural dynamics of PSII RCs.

KEYWORDS

chlorophyll-a fluorescence, core complex of photosystem II, protonation, rate-limiting step, structural dynamics, waiting time

1 Introduction

Photosystem II (PSII) is a large multi-subunit pigment-protein complex embedded in the thylakoid membranes of oxygenic photosynthetic organisms. It plays a fundamental role in light-driven electron transfer, initiating the process of water splitting and oxygen evolution (Barber, 2004; Cardona et al., 2012). PSII catalyses the extraction of electrons from water molecules at the oxygen-evolving complex (OEC) – a process which ultimately supports photosynthetic carbon fixation (Nelson and Yocum, 2006) – using light energy absorbed by its pigment-protein antenna system (Shen, 2015). The catalytic activity of PSII, including charge separation, electron transfer, and water oxidation, relies on finely tuned protonation and deprotonation events that are intricately coupled to its redox chemistry. Altogether, PSII represents the central engine of oxygenic photosynthesis, converting light energy into chemical potential by coupling water oxidation to quinone reduction, thus fuelling life on Earth (Barber, 2004; Shevela et al., 2023).

PSII is organized as a homodimeric supercomplex, where each monomer contains a core complex (CC) composed of several protein subunits and cofactors essential for its function. The reaction centre (RC) is formed by the D1 and D2 proteins, which coordinate all components necessary for the primary charge separation. The integral antenna proteins CP43 and CP47 bind chlorophyll-*a* (Chl-*a*) and β -carotene molecules that capture and funnel light energy to the RC. PSII also contains the α and β subunits of cytochrome b_{559} , numerous low-molecular-weight proteins, and the OEC (Umena et al., 2011; Shen, 2015; Suga et al., 2019; Shevela et al., 2023). The RC of PSII contains four Chl-*a* molecules (a “special pair” forming P_{680} , and two accessory Chls), two pheophytins (Pheo), a non-heme iron, and two plastoquinone molecules (Q_A and Q_B), organized along two pseudo-symmetrical branches. Redox-active tyrosine residues (Y_Z and Y_D) facilitate electron transfer and connect the RC to the donor side of PSII. The assembly and activity of the OEC are also closely associated with CP43 (Shen, 2015; Suga et al., 2019; Zabret et al., 2021).

The charge separation process is initiated upon illumination of the dark-adapted, open-state PSII ($PSII_O$), leading to the formation of the radical pair $P_{680}^+Pheo^-$ (Romero et al., 2017). This is followed by rapid electron transfer from $Pheo^-$ to the primary quinone acceptor Q_A , stabilizing the charge-separated state ($P_{680}^+Q_A^-$). P_{680}^+ is then re-reduced by Y_Z , which in turn oxidizes the Mn_4CaO_5 cluster of the OEC, advancing the catalytic cycle of water oxidation and generating the S_2 state of the OEC. Following these primary reactions, the PSII centres are found in closed state ($PSII_C$), with all Q_A reduced. Subsequent electron transfer from Q_A^- to the secondary quinone Q_B typically occurs over several hundred microseconds under physiological conditions (Shlyk-Kerner et al., 2006). This step can be blocked by herbicides, such as 3-(3',4'-dichlorophenyl)-1,1'-dimethylurea (DCMU or diuron), which prevent electron flow beyond Q_A , leading to the stabilization of the charge-separated state. In the presence of DCMU, the lifetime of $PSII_C$ is significantly extended and dominated by charge recombination between Q_A^- and the oxidized OEC (S_2) (Tyystjarvi and Vass, 2004).

The redox potential of these cofactors is sensitive to various environmental factors, particularly to the protonation state of

surrounding amino acid residues and water molecules. Protonation and deprotonation reactions within PSII are essential for maintaining the proton-coupled electron transfer that drives the catalytic cycle of water oxidation (Siegbahn, 2013; Zaharieva and Dau, 2019). These processes are mediated by complex hydrogen-bonding networks and proton transfer pathways, that link the bulk aqueous phase to the OEC and the quinone sites near and beyond the Q_B pocket (Saito et al., 2011; Umena et al., 2011; Kaur et al., 2021; Sugo et al., 2022; Li et al., 2024; Yano et al., 2024). These protonation-dependent processes are also reflected in variable Chl-*a* fluorescence (Crofts and Wraight, 1983; Krieger-Liszak et al., 2008; Vinyard et al., 2013).

Chl-*a* fluorescence induction kinetics (ChlF) is one of the most widely used non-invasive techniques to monitor the functioning of PSII and oxygenic photosynthesis across a broad range of biological systems (Govindjee and Papageorgiou, 2004; Papageorgiou and Govindjee, 2011). ChlF induction kinetics provide critical information on electron transfer reactions, PSII activity, plastoquinone redox state, photoprotection, state transitions, responses to environmental stresses, and mechanisms of photoinhibition and repair (Schreiber et al., 1995; Horton and Ruban, 2005; Prášil et al., 2018).

Traditionally, ChlF induction curves – characterized by a rise from the minimal fluorescence level (F_o) to the maximum (F_m) – were interpreted within the widely accepted Q_A model. According to this model, the variable fluorescence ($F_v = F_m - F_o$) arises exclusively from the reduction of the primary quinone electron acceptor Q_A in PSII (Duysens and Sweers, 1963; Strasser et al., 2004; Stirbet and Govindjee, 2012). The multiphasic O–J–I–P fast fluorescence rise is thus ascribed to the progressive closure of PSII reaction centres via Q_A reduction, while the sigmoidal shape of the O–P rise in the presence of DCMU is interpreted as a manifestation of energetic connectivity between PSII units (Joliot and Joliot, 1964; Lavergne and Trissl, 1995; Stirbet, 2013). However, despite the model's widespread application, accumulating evidence has highlighted fundamental controversies and limitations of the model (Joliot and Joliot, 1979; Vredenberg, 2011; Schansker et al., 2014; Magyar et al., 2018; Laisk and Oja, 2020). Notably, it has been demonstrated that in DCMU-treated samples, the F_1 fluorescence level following the first single-turnover saturating flash (STSF) is significantly lower than F_m , and that F_m cannot be attained solely through Q_A reduction, induced by the first STSF, but rather requires multiple excitations (Joliot and Joliot, 1979) and sufficiently long $\Delta\tau$ waiting times between the flashes (Magyar et al., 2018). The requirement of $\Delta\tau$ between STSFs indicated the involvement of rate-limiting steps in the dark-to-light transition of PSII.

Our earlier experiments of Chl-*a* fluorescence transients elicited by trains of STSFs revealed the role of subtle conformational changes and suggested the involvement of dielectric relaxation processes (Sipka et al., 2019; Sipka et al., 2021), as proposed earlier for the formation of the light-adapted charge-separated state of purple bacterial RCs (Malferrari et al., 2013; Sipka et al., 2022). These processes govern the transition of PSII from its closed state ($PSII_C$) to a light-adapted charge-separated state ($PSII_L$), which is characterized by enhanced stabilization of charges compared to $PSII_C$ (Sipka et al., 2021). We also showed that the $\Delta\tau_{1/2}$ half-waiting times and the stepwise fluorescence increments, $F_1-F_2-F_3-\dots-F_m$, depended on the lipid composition of the RC matrix and the

temperature (Magyar et al., 2022; Magyar et al., 2023; Magyar et al., 2024). However, our understanding concerning the exact physical mechanisms underlying the dark-to-light transition of PSII RC and the molecular environmental factors on which these transitions depend are still rudimentary. In particular, the pH dependence of these transitions has not been studied – although numerous investigations showed that variations in the pH significantly influence the structure and function of PSII. Acidic pH has been shown to accelerate donor-side photoinhibition, impair electron transfer from the OEC, and lead to altered fragmentation patterns of the D1 protein, often accompanied by the formation of hydroxyl radicals (Hideg et al., 1994; Spetea et al., 1997; Krieger-Liszkay et al., 2008). Low pH also facilitates the dissociation of extrinsic proteins (PsbO, PsbP, and PsbQ), leading to inactivation of the OEC (Shen and Inoue, 1991). It has been demonstrated that at an acidic pH of 3.0, the water oxidation activity declines, accompanied by release of Ca^{2+} and Mn ions essential for OEC stability (Ono and Inoue, 1988). pH modulates S-state transitions, especially those beyond S_2 , thereby affecting the efficiency of the water-splitting cycle (Bernat et al., 2002; Suzuki et al., 2005). The pH dependence of oxygen evolution in native PSII membranes is bell-shaped, with maximum at around pH of 6.5 (Vass and Styring, 1991; Semin et al., 2015). Further, protonophores such as CCCP (carbonyl cyanide m-chlorophenyl hydrazone) and FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone), which disrupt transmembrane proton gradients (Heytler and Prichard, 1962; Volkov et al., 1998), act as ADPR reagents, accelerating the deactivation reactions of the OEC (Renger, 1972). Remarkably, the photoreduction of Q_A has been shown to be largely insensitive to the variations of pH between 6.0 and 8.0 (Demeter et al., 1985; Krieger et al., 1995).

In an earlier work, we hypothesized that the gradual formation of PSII_L is governed by dielectric relaxation processes in the RC matrix, due to the presence of polarizable groups, protonatable residues and bound water molecules, at the donor side of the RC (Sipka et al., 2022). Here we tested this hypothesis by investigating the pH dependence of $\Delta\tau_{1/2}$ and the F_1 level of ChlF, in the range between pH 5.0 and 8.0, on DCMU-treated PSII CC isolated from *Thermotichus* (*T.*) *vulcanus*. Our experiments, performed at different temperatures (5, –20 and –60°C) and in the presence and absence of FCCP or CCCP, provide evidence on the role of protonation/deprotonation processes in the structural dynamics of PSII RCs and in the gradual formation of PSII_L , in particular.

2 Materials and methods

2.1 Source material

A thermophilic cyanobacterial strain, *T. vulcanus*, originally isolated from a hot spring in Yunomine, Japan (Koike and Inoue, 1983), was used in this study. Cells were cultivated photoautotrophically in batch cultures using BG11 medium (pH 7.0) at 50°C, under continuous white fluorescent light of 50–100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ photon flux density (Shen et al., 2011). Cultures were maintained with constant aeration on a gyratory shaker operating at 100 rpm.

2.2 Sample preparation

PSII CCs of *T. vulcanus* were isolated following previously described protocols (Shen and Inoue, 1993; Shen and Kamiya, 2000; Shen et al., 2011; Kawakami and Shen, 2018). In the final purification steps, dimers and monomers were separated from crude PSII using an anion exchange column. The column buffer contained 30 mM MES-NaOH (pH 6.0), 3 mM CaCl_2 , and 0.03% β -DDM, and a linear gradient ranging from 170 to 300 mM NaCl was applied. After collecting the monomeric and dimeric fractions, polyethylene glycol (PEG) 1,450 was added to a final concentration of 13%, followed by centrifugation to precipitate and concentrate the samples. The resulting pellets were resuspended in a buffer containing 30 mM MES-NaOH (pH 6.0), 20 mM NaCl, and 3 mM CaCl_2 and stored in liquid nitrogen or at –80°C until further use. For spectroscopic analyses, isolated PSII CCs were diluted in a reaction buffer containing 5% glycerol, 20 mM MES (pH 6.0), 20 mM NaCl and 3 mM CaCl_2 .

2.3 Chlorophyll-a fluorescence transients

Chl-*a* fluorescence transients were recorded following a dark adaptation period of 5 min at room temperature, followed by an additional 5 min dark adaptation at the given temperatures, on which the measurements were performed.

For PAM measurements, the Chl concentration of PSII CC was diluted to $\sim 10 \mu\text{g mL}^{-1}$ in the reaction medium, and to $\sim 20 \mu\text{g mL}^{-1}$ in case of the fast fluorescence measurements. DCMU, dissolved in dimethyl sulfoxide (DMSO) was added to all samples immediately before the measurements to a final concentration of 40 μM (with final DMSO concentration kept below 1%). CCCP, also dissolved in DMSO, was added to a final concentration of 5 μM ; FCCP, dissolved in ethanol, was used at a final concentration of 5 μM (unless stated otherwise in the text).

2.3.1 PAM fluorescence

Chl-*a* fluorescence was measured using a Multi-Color PAM (Phase Amplitude Modulation) fluorometer (MC-PAM; Walz, Effeltrich, Germany). Fluorescence transients were induced by STSFs generated by a xenon flash unit (Excelitas LS-1130-3 Flashpac with FX-1163 Flashtube with reflector, Wiesbaden, Germany) with a pulse duration of 1.5 μs duration at half-peak intensity. The modulated measuring light was applied at low intensity, non-actinic level, with a frequency of 1 kHz, which limited the temporal resolution of the fluorescence kinetics to several milliseconds. Samples were placed on the sample holder of a thermoluminescence device for temperature control. Flash timing was precisely managed using a digital pulse generator (525 Six Channel Digital Pulse/Delay Generator, Berkeley Nucleonics, California, United States), and kinetic traces were acquired using the MC-PAM's dedicated software.

The increments of Chl-*a* fluorescence from F_1 -to- F_2 were measured by applying a second STSF at varying delay times ($\Delta\tau$) after the first flash. These fluorescence increments were normalized to the maximum fluorescence level (F_m), determined using multiple-turnover saturating laser flashes. The normalized values $(F_{1,2}-F_0)/(F_\text{m}-F_0)$, were plotted as a function of $\Delta\tau$. The half-rise times ($\Delta\tau_{1/2}$), representing the $\Delta\tau$ at which 50% of the maximum F_1 -to- F_2 increment is reached, were calculated using a logistic fitting function.

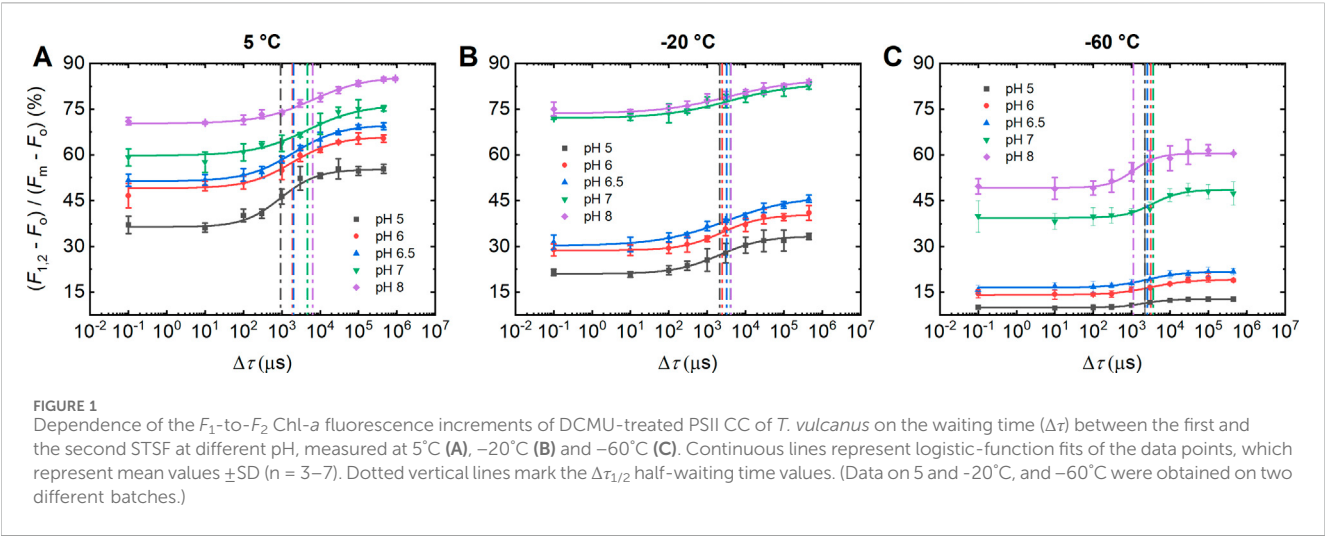


FIGURE 1 Dependence of the F_1 -to- F_2 Chl-*a* fluorescence increments of DCMU-treated PSII CC of *T. vulcanus* on the waiting time ($\Delta\tau$) between the first and the second STSF at different pH, measured at 5°C (A), -20°C (B) and -60°C (C). Continuous lines represent logistic-function fits of the data points, which represent mean values $\pm$ SD ($n = 3-7$). Dotted vertical lines mark the $\Delta\tau_{1/2}$ half-waiting time values. (Data on 5 and -20°C, and -60°C were obtained on two different batches.)

TABLE 1 Dependences of double-STSF induced F_1 -to- F_2 $\Delta\tau_{1/2}$ half-rise times of DCMU-treated PSII CC of *T. vulcanus* at different pHs and temperatures, using the data in Figure 1. The 2 sets of data at -20°C indicate the results of two parallel measurements on two different batches.

Temperature (°C)	pH 5	pH 6	pH 6.5	pH 7	pH 8
5°C	1.0 $\pm$ 0.2 m	1.9 $\pm$ 0.5 m	2.0 $\pm$ 0.2 m	4.6 $\pm$ 1.8 m	6.3 $\pm$ 1.0 m
-20°C	2.1 $\pm$ 0.5 m	2.4 $\pm$ 0.4 m	3.1 $\pm$ 1.1 m	3.9 $\pm$ 1.5 m	4.0 $\pm$ 1.4 m
		2.1 $\pm$ 0.2 m	3.2 $\pm$ 1.8 m	4.4 $\pm$ 2.1 m	1.7 $\pm$ 0.6 m
-60°C	2.1 $\pm$ 0.1 m	3.2 $\pm$ 0.9 m	2.6 $\pm$ 0.8 m	3.6 $\pm$ 1.3 m	1.1 $\pm$ 0.3 m

2.3.2 Fast Chl fluorescence measurements

Fast fluorescence transient measurements were performed using a Handy-PEA fluorometer (Hansatech Instruments Ltd., UK), with an excitation photon flux density of 3,380 μ mol photons $m^{-2} s^{-1}$. The resulting fluorescence induction curves were analysed and fitted using a sum of three exponentials.

2.4 Gel electrophoresis

The integrity of PSII CC dimers at different pHs was checked by blue native polyacrylamide gel electrophoresis on a 5%–13% (w/v) gradient gel at 6°C according to (Schagger et al., 1994). The amount of samples loaded on each lane was 0.4 μ g of Chl. The native gel was stained with Coomassie Blue G-250.

3 Results

To elucidate the role of polarizable groups and protonatable residues in the gradual, light-induced formation of PSII_L and the structural dynamic features of the RC matrix in charge-separated state, we performed STSF-induced and fast Chl-*a* fluorescence measurements on isolated DCMU-treated PSII CCs at various protonation states, via adjusting the pH of the reaction medium between 5.0 and 8.0 and using protonophores. Our major aim was to determine the pH-dependence of the ChlF fluorescence parameter $\Delta\tau_{1/2}$, which has been shown to be correlated with the structural dynamics of closed PSII RCs (see

Introduction). In addition, we also monitored the response of other ChlF parameters to variations in the pH and the temperature.

3.1 pH dependence of $\Delta\tau_{1/2}$ of the F_1 -to- F_2 transition

To probe the effect of pH, between 5.0 and 8.0, on $\Delta\tau_{1/2}$, associated with the F_1 -to- F_2 increment of ChlF, we performed double-STSF induced ChlF experiments on DCMU-treated PSII CC of *T. vulcanus* at different temperatures, 5, -20 and -60°C. Figure 1A shows that, at 5°C, the $\Delta\tau_{1/2}$ half-waiting time values, marked with dashed vertical lines, exhibited characteristic pH dependence: moderate and strong increases of $\Delta\tau_{1/2}$ in the pH ranges between 5.0 and 6.5, and between 6.5 and 8.0, respectively. As shown in Figures 1B,C and Table 1, the pH dependence of $\Delta\tau_{1/2}$ is less pronounced at cryogenic temperatures. These data, on one hand, are in harmony with the proposed role of protonatable residues in the gradual formation of PSII_L, and indicate, on the other hand, that the protonation-dependent reorganizations are hindered at low temperatures.

Note that Table 1 contains two data sets at -20°C, obtained on two different batches of PSII CC. These data show that different batches display similar trends of their pH dependences in the physiologically relevant pH range, between 6.0 and 7.0, but the actual $\Delta\tau_{1/2}$ values vary from batch-to-batch (Supplementary Figure S1). Note that Supplementary Table S1 also displays the corresponding P parameter of the logistic fit function and the F_v/F_m values, which testify that the

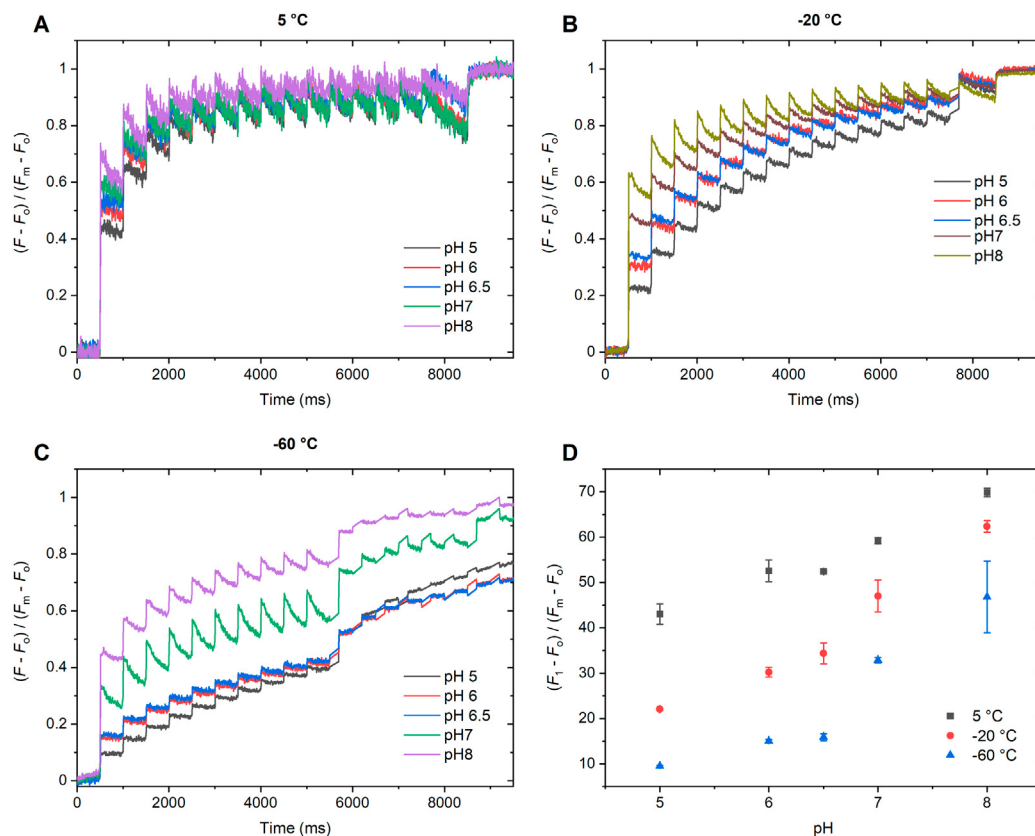


FIGURE 2
pH-dependent variations of the STSF-induced Chl-a fluorescence transients of DCMU-treated PSII CC of *T. vulcanus* at 5°C (A), -20°C (B) and -60°C (C). (Each transient represents the average obtained from 3 independent experiments on the same batch; data on 5 and -20, and -60°C were obtained on two different batches). Panel (D) depicts the dependence of the STSF-induced F_1 fluorescence level on the pH at different temperatures. (For a more detailed pH dependence at -20°C between 6.5 and 7.0, see [Supplementary Figure S4](#)).

functional activity of PSII, including photochemistry and structural dynamics, is retained in all samples and under all experimental conditions used here. To test the stability of PSII CC at different pHs between pH 5.0 and 8.0, we performed Blue Native PAGE, which revealed no instability of the complexes ([Supplementary Figure S2](#)).

It is an interesting, unexpected finding of our experiments with double STSFs that the magnitude of the F_1 fluorescence increment exhibited a strong dependence on the pH of the reaction medium ([Figure 1](#)). These changes were more pronounced at cryogenic temperatures, which consistently indicated that the largest changes occurred upon increasing the pH from 6.5 to 7.0.

3.2 pH dependence of ChlF amplitudes induced by trains of STSFs at different temperatures

To further investigate the pH-dependence of the F_1 fluorescence level of ChlF and to test the behavior of the additional increments, beyond the F_0 -to- F_1 rise, associated with the PSII_O-to-PSII_C, we conducted ChlF measurements at different temperatures, using trains of STSFs. As can be seen in [Figure 2](#), F_1 increases in a stepwise manner with gradually increasing the pH between 5.0 and 8.0. These data show that the transitions from PSII_C-to-

PSII_L in all cases proceed through distinct, quantifiable steps and indicate that the protonation state of key residues within PSII modulates the charge stabilization and recombination processes and possibly the structural dynamics of the RCs.

As in [Figure 1](#), the variations were more prominent by lowering the temperature. Earlier, we reported similar temperature dependent variations in the F_0 -to- F_1 rise in DCMU-treated PSII CC of *T. vulcanus* at pH 6.0 and in spinach thylakoid membranes at pH 7.5 ([Magyar et al., 2023](#)). In general, the temperature dependence of the transients recorded on PSII CC at different pHs ([Figures 2A–C](#)) followed similar trends as reported earlier for pH 6.0 ([Magyar et al., 2023](#)).

It was interesting to observe that, similar to the pH dependence of $\Delta\tau_{1/2}$ shown above ([Figure 1](#)), the largest pH-dependent changes occurred in the narrow range between 6.5 and 7.0. This may indicate the role of a (few) specific residue(s) with pK value(s) in this range. The sharp pH dependence can most prominently be seen at cryogenic temperatures, most strongly at -60°C ([Figures 2C,D](#)). It is equally interesting to note that similar, albeit less marked pH dependences are present in the F_1 -to- F_2 increments of ChlF ([Supplementary Figure S3](#)). This pH dependence of the increments appeared to persist throughout the entire transient, as it can be inferred from the pH dependences of the number of STSFs required to reach the 50% and 80% of the F_1 -to- F_m rise at -20°C and 30% at -60°C ([Supplementary Figure S3](#)).

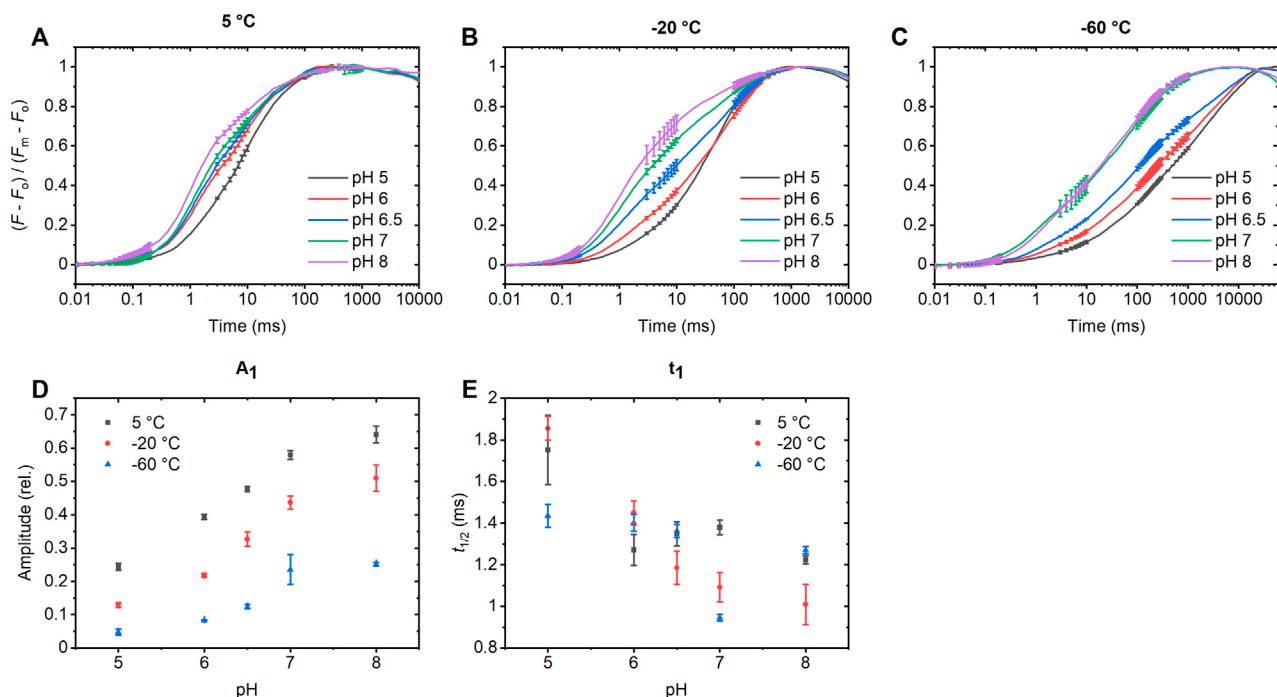


FIGURE 3
Fast Chl-*a* fluorescence rise of DCMU-treated PSII CCs of *T. vulcanus* at 5°C (A), -20°C (B) and -60°C (C), normalized to $(F_m - F_0)$. The curves were fitted with 3 exponentials and the amplitudes (D) and half-rise times (E) of the first component, representing the initial rise, were plotted. In case of the -60°C measurement, the curve was fitted between 50 μ s and 700 ms. The photon flux density of the excitation was 3,380 mmol photons $m^{-2} s^{-1}$. (Since according to the experiments using STSFs, the rises are composed by more than three exponentials, we do not plot the pH dependences of the second and third exponentials.)

3.3 pH dependences as reflected by the fast fluorescence transients

We recorded fast Chl-*a* fluorescence transients, under comparable experimental conditions as for STSFs (Sections 1 and 2), using the well-established, widely used technique of fast Chl-*a* fluorescence transients. In the presence and absence of DCMU, these transients, elicited by intense long flashes, are commonly termed as O-P and O-J-I-P transients, respectively (see Introduction).

It will be shown below that fast ChlF traces reflect the basic attributes of the pH and temperature dependence of the PSII_O-PSII_C-PSII_I transitions. However, it must be emphasized that these experiments are not suited for the determination of the two key parameters of ChlF, $\Delta\tau_{1/2}$, related to the structural dynamics of PSII, and the magnitude of F_1 level, associated with the formation of PSII_C. Nevertheless, these are presented because double-STSF-induced PAM fluorescence measurements and adjustable $\Delta\tau$ waiting times between STSFs, to our knowledge, are not used in other laboratories, and no standardized protocol is currently available for commercially available Chl-*a* fluorimeters.

As shown in Figures 3A–C, fast ChlF transient measurements on DCMU-treated PSII CC revealed – qualitatively – similar pH and temperature dependent changes as shown above with STSF-induced transients. By fitting the fluorescence transients with a three-exponential model and analyzing the amplitudes and rise-times, we observed a clear trend in the first component – which is evidently most closely correlated with the F_1 rise (PSII_O-PSII_C). It can be seen

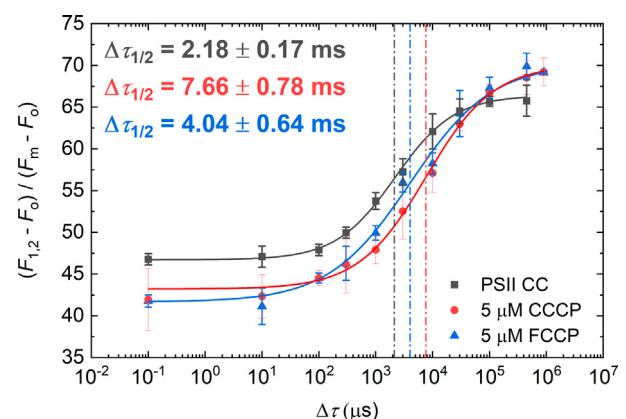
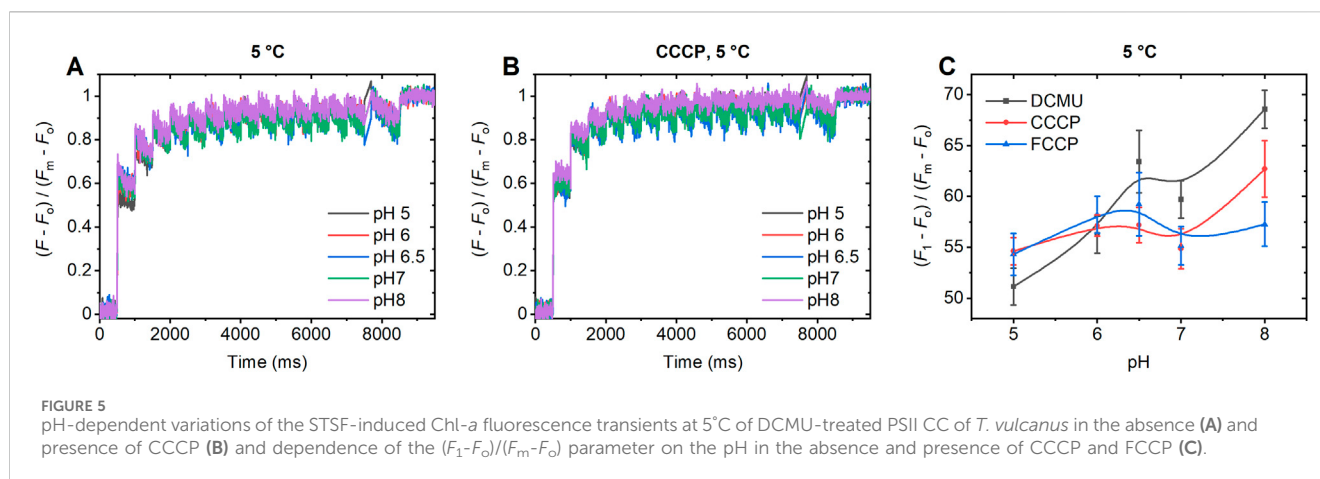


FIGURE 4
Dependence of the F_1 -to- F_2 Chl-*a* fluorescence increments of DCMU-treated PSII CC at pH 6.0 on the waiting time ($\Delta\tau$) between the first and the second STSF in the presence of 5 μ M CCCP and FCCP. Continuous lines represent logistic-function fits of the data points, which represent mean values $\pm$ SD ($n = 3-5$). Dotted vertical lines mark the $\Delta\tau_{1/2}$ half-waiting time values.

that, in harmony with the data in Figures 1, 2, the amplitude of this component increased with rising the pH between 5.0 and 8.0, while the rise-times became shorter (Figures 3D,E). The gradual increase of the amplitude of the first exponential with gradually increasing the pH, agrees well with the data obtained with STSF-induced ChlF data (Figure 2D). In addition, the fast ChlF measurements revealed



that the rate of F_0 -to- F_1 rise, induced by a continuous light (long flash), also depends heavily on the pH. Evidently, the rise kinetics of F_1 at different pHs and temperatures could and should be determined using STSF excitation.

3.4 Effect of protonophores

The results of ChlF measurements obtained at different pHs strongly suggest that the structural dynamics PSII RCs depend heavily on polarizability and state of protonation of certain residues of the RC matrix. To substantiate this relationship, we examined the effects of protonophores. To this end, we tested the effects of CCCP and FCCP on the $\Delta\tau_{1/2}$ parameter and on the F_0 -to- F_1 rise of ChlF in DCMU-treated PSII CC. These protonophores are known to disrupt proton gradients across membranes by facilitating proton leakage. In the absence of a membrane system, as in our case, they might interact directly with protons within the PSII CC.

When assessing the effect of CCCP and FCCP on the $\Delta\tau_{1/2}$, we found that they significantly increased the half-waiting time (Figure 4) to values reaching levels comparable to those observed in PSII CC at pH 7.0 and 8.0 at 5°C (cf. Table 1). This suggests that their presence alters the protonation dynamics within PSII, mimicking a higher pH environment. Although CCCP and FCCP are weak acids, they act as proton carriers, binding and releasing protons depending on the local protonation equilibrium (Benz and McLaughlin, 1983). It is shown in Supplementary Figure S5 that FCCP at 2 and 5 μM exerted similar effects, suggesting that the protonophores act at low concentrations.

As shown in Figure 5, CCCP and FCCP largely eliminated the pH-dependent variations of the F_1 level of ChlF at 5°C. (For the effect of FCCP on the kinetic transients, see Supplementary Figure S6). These results corroborate our conclusion on the role of light-induced proton motions inside PSII CC by these uncouplers. (Note that all these effects are observed in the presence of DCMU, which permits only a single stable light-induced charge separation.) In contrast to their effects at 5°C, at -20°C (Supplementary Figure S7), CCCP and FCCP had no discernible effect on the F_1 level, suggesting that their activity, based on their ability to shuttle protons between different lipophilic sites

(Kasianowicz et al., 1984; Lim et al., 2001; Pansook et al., 2019), is halted at cryogenic temperatures. This is evidently a consequence of the increased rigidity and lack of fluidity in the surrounding environment, which hinders the mobility and proton-shuttling activity of the protonophores (Hazel, 1995; Monteiro et al., 2011).

It is interesting to remark that no significant changes in the fluorescence transients were observed upon treatment of the samples with NH_4Cl or nigericin (either in the presence or absence of K^+ ions), when tested at pH 6 (Supplementary Figure S8). Despite their known effects on proton transport and pH modulation, neither compound substantially affected the multi-STSF fluorescence transients in PSII CC. This might be explained by their limited accessibility to specific sites involved in the protonation/deprotonation processes.

4 Discussion

The major aim of this work was to test the hypothesis set in our previous work, on the role of polarizable groups at the donor side of PSII in the gradual formation of PSII_L , the light-induced charge-separated state of the RC of PSII, and in the structural dynamics of the RC matrix, in general (Sipka et al., 2022). The processes involved in the formation of PSII_L are of substantial interest because of the enhanced stabilization of charges in PSII_L compared to PSII_C (Sipka et al., 2021). Our hypothesis on the mechanism of formation of PSII_L was largely inspired by the experimental findings of Allen et al. (2023), who used wild-type and mutant purple bacterial RCs (bRCs) to demonstrate a strong correlation between the stabilization of charges in the light adapted state of the bRC and polarizable amino acid residues near its primary special pair (P) molecules. Data presented by these authors pointed to the involvement of a hydrogen-bond network and bound water molecules, forming a proton transfer pathway around P.

Our investigations provide experimental evidence for the role of protonation/deprotonation processes in the gradual formation of PSII_L . In particular, we have shown that the $\Delta\tau_{1/2}$ half-waiting time, a key parameter characteristic of the stepwise light-induced PSII_C -to- PSII_L transition, depends strongly on the pH of the reaction medium. Here, $\Delta\tau_{1/2}$ was determined for the first step of

this transition, manifested in the F_1 -to- F_2 Chl-*a* fluorescence level increment (Figure 1). (Earlier we have shown that $\Delta\tau_{1/2}$ is involved in all later steps tested during the F_v induction period (Magyar et al., 2023).) It was interesting to observe that upon increasing the pH from 6.5 to 7.0 and 8.0, the value of $\Delta\tau_{1/2}$ at 5 °C dramatically increased (Table 1). These data indicate that deprotonation of some residues, with pK between 6.5 and 7.0, strongly hampers the subtle structural reorganization occurring in the RC matrix. This conclusion was substantiated by the effect of the protonophores CCCP and FCCP, which led to increased $\Delta\tau_{1/2}$ values, in a similar manner as elevating the pH from 6.5 to 7.0 (Figures 4, 5). This effect of protonophores is most likely related to their ability to accelerate the deactivation reactions of the OEC, similar to other ADPR reagents (Renger, 1972). They act as mobile proton carriers and can interact with PSII by altering the local availability of protons, disrupting structured water networks, and affecting the protonation state of residues involved in proton-coupled electron transfer (Shioi et al., 1976; Nemson and Melis, 1990; Samuilov and Barsky, 1993). By disrupting structured water networks and protonation states of key residues, protonophores provide insights into the electrostatic environment of PSII. It is well known that CCCP modifies the donor side reactions of PSII (Homann, 1971; Renger, 1972). Although these protonophores are acidic, their function is not dictated by a static proton load; rather, they dynamically bind and release protons depending on the surrounding pH gradient. However, the exact site of the effect of these protonophores and the identity of the residues, with pK values between 6.5 and 7.0, remain to be clarified.

In addition to the marked pH dependence of $\Delta\tau_{1/2}$, we also found, unexpectedly, that the F_1 level of the Chl-*a* fluorescence increased dramatically upon increasing the pH between 5.0 and 8.0; again, the increase was most prominent in the narrow pH range between 6.5 and 7.0 (Figures 1, 2). It is noteworthy that the overall pattern of ChlF transients remained the same at all pHs and temperatures. All transients displayed $F_1 < F_m$ and to reach F_m , multiple STSFs (or multi-turnover flashes) were required. Furthermore, all samples under all conditions possessed high F_v/F_m values (about equal or larger than 0.8 – see Supplementary Table S1). This suggests that the pH and temperature dependences of the F_o -to- F_1 rise and the $\Delta\tau_{1/2}$ half-waiting time might be governed by similar physical mechanism(s), both containing protonation-dependent variations in the structural dynamics of PSII_C. The enhanced F_o -to- F_1 rises at high pHs might, in large part, reflect increased levels of the structural plasticity of the RCs; at lower pHs, similar increments can only be induced by additional STSFs (Figure 2, see also Supplementary Figure S4). In this context, it is important to recall that Oja and Laik (2020) reported a ‘prompt’ rise to approximately $1.8 \times F_o$; this further increased to $\sim 3 \times F_o$ within the sub-ms time range, an increment attributed to protein conformational change. Indeed, at low pH and low temperatures, we detected fluorescence levels reaching only $\sim 1.8 \times F_o$ or lower – with ms time resolution (Figures 1, 2, see also Magyar et al. (2018); Sipka et al. (2021)). As concluded from measurements at cryogenic temperatures, the rise associated with closing the RC, *i.e.*, the reduction of Q_A was considerably smaller than $1.8 \times F_o$ (Schansker et al., 2011; Sipka et al., 2021). In our experiments, at pH 6.0 and -60°C , we reach a quasi-steady state level of $1.13 \times F_o$ (corresponding to 15% F_v rise – see Figure 1C). It is important to recall that the photoreduction of Q_A between pH 6.0 and 8.0 is essentially pH independent (Demeter et al., 1985; Krieger et al., 1995), and occurs

at cryogenic temperatures (Kirmaier et al., 1985; Yakovlev and Shuvalov, 2016). These data will aid us to elucidate the physical mechanisms associated with F_v , in general.

The origin of the pH and temperature dependent variations of the F_1 level, in particular, remains to be clarified. To this end, it would be important to perform sub-ms time resolution experiments at different temperatures and pHs on DCMU-treated PSII CC. Using PAM fluorimetry, sub-ms (and even sub- μ s) fluorescence components have recently been recorded in *Chlorella* cells (Klughhammer et al., 2024). Sub-ms time resolution PAM experiments on DCMU-treated PSII CC, under comparable conditions as applied here, are in preparation in our laboratory. However, they are outside the scope of the present paper, which focuses on the role of protonation/deprotonation processes in the gradual formation of PSII_L. In the present study, sub-ms time resolution measurements are confined to the so-called fast Chl-*a*, O-P type of, fluorescence transients, using intense long pulses. These data revealed elevated levels and rates of the first exponential rise component of the transient (Figure 3). Hence, they support the notion that the fast rise of ChlF upon the first STSF, *i.e.*, the quasi-stationary F_1 level, depends to a large extent on the structural plasticity of PSII_C. The nature of the (most probably subtle) reorganizations at the donor side of PSII RC, marked by the fast transients in ChlF, and the role of the protonation/deprotonation processes, in particular, remain to be clarified.

The enhanced stabilization of charges during PSII_O-to-PSII_L has been proposed to follow a mechanism similar to that in bRCs, with dielectric relaxation processes optimizing the RC matrix’s dielectric properties. Dielectric relaxation processes have earlier been proposed to play important roles in the formation of light adapted states of bRC and PSII (Malferrari et al., 2013; Sipka et al., 2021). Here we established the involvement of protonatable/deprotonatable groups in modulating F_v increments, which are thus thought to be linked to conformational changes and dielectric relaxation. In broad terms, the S-state dependent proton release and fluorescence oscillation patterns, reported by Delosme and Joliet (2002) can be reconciled with our observations—with the assumption that the protonation/deprotonation processes are not confined to the OEC but extend to the donor side of the RC. In general, protonation and deprotonation reactions within PSII are critical for proton-coupled electron transfer (PCET), involving amino acid residues such as histidines and carboxylates that form hydrogen-bonded networks (Saito et al., 2011; Vogt et al., 2015). In particular, residues located near Y_Z (D1-Tyr161) and the non-heme iron–bicarbonate site modulate the protonation state of redox intermediates like P_{680}^+ , Q_A^- , and $Y_Z^\bullet$, thereby influencing PSII activity and recombination kinetics (Ferreira et al., 2004). Redox-active tyrosines, Y_Z and Y_D , also exhibit distinct pH-dependent reaction rates (Moser et al., 1995; Reece et al., 2006), further influencing PCET efficiency. However, it is important to note that in our case PCET does not apply, due to the single turnover stable charge separation and the formation of the closed state of the PSII RC upon the first STSF.

Although the pH dependent variations in F_v parameters can be explained with conformational changes that are influenced by protonation states of key residues, alternative mechanisms may contribute to the observed changes. For instance, shifts in charge recombination kinetics and/or an increased fraction of inefficient centers may affect the kinetic traces (Laverne and Rappaport, 1998).

Indeed, some pH-dependent recombination-associated decays are observed at high pH after the first flash (Figure 2); however, the observed magnitudes and rates offer no explanation for the basic features of the variations either in $\Delta\tau_{1/2}$ or in the F_1 level. Instead, the decays observed after multi-STSF excitation likely stem from the inherent plasticity of PSII rather than $S_2Q_A^-$ recombination (Magyar et al., 2018). It is also to be noted that the half-time for $S_2Q_A^-$ recombination is 1–2 s, with pH shifts affecting the equilibrium between S_2P_{680} and $S_1P_{680}^+$ (Vass and Styring, 1991) – but they remain within the range of our time resolution. Also, the required waiting times and the high F_v/F_m values essentially rule out the presence of inactive centers in sizeable amounts; this holds true also for photoinhibition, which is known to be enhanced under low pH due to destabilization of the OEC (Debus, 1992).

Together, these findings underscore the complex interplay between polarizable groups and protonatable residues, redox processes, and subtle structural reorganizations in PSII. Understanding how these factors are modulated under varying pH conditions and in the presence of protonophores is essential for elucidating the mechanisms underlying water oxidation, photoprotection, and photodamage. These results also raise important questions about the more precise mechanistic origin of F_v , in molecular terms, the role of electric field formation, conformational adjustments, and protein-lipid interactions in PSII function. Addressing these questions is crucial not only for understanding fundamental photosynthetic mechanisms but also for improving the interpretation of Chl fluorescence data across all scales – from molecular studies to ecological monitoring.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

MM: Writing – original draft, Formal Analysis, Visualization, Methodology, Project administration, Investigation, Data curation, Conceptualization, Validation. GS: Writing – review and editing, Conceptualization, Methodology, Software. ID: Formal Analysis, Visualization, Writing – review and editing. XC: Resources, Writing – review and editing. XW: Resources, Writing – review and editing. GH: Writing – review and editing, Resources, Funding acquisition. J-RS: Funding acquisition, Resources, Writing – review and editing. PL: Funding acquisition, Project administration, Writing – review and editing, Supervision. GG: Supervision,

Conceptualization, Writing – original draft, Project administration, Funding acquisition.

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphbi.2025.1623224/full#supplementary-material>

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