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Acceptor and donor-side interactions of phenolic inhibitors in Photosystem II

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Abstract

Certain phenolic compounds represent a distinct class of Photosystem (PS) II Q_B site inhibitors. In this paper, we report a detailed study of the effects of 2,4,6-trinitrophenol (TNP) and other phenolic inhibitors, bromoxynil and dinoseb, on PS II energetics. In intact PS II, phenolic inhibitors bound to only 90–95% of Q_B sites even at saturating concentrations. The remaining PS II reaction centers (5–10%) showed modified Q_A to Q_B electron transfer but were sensitive to urea/triazine inhibitors. The binding of phenolic inhibitors was 30- to 300-fold slower than the urea/triazine class of Q_B site inhibitors, DCMU and atrazine. In the sensitive centers, the $S_2Q_A^-$ state was 10-fold less stable in the presence of phenolic inhibitors than the urea/triazine herbicides. In addition, the binding affinity of phenolic herbicides was decreased 10-fold in the $S_2Q_A^-$ state than the S_1Q_A state. However, removal of the oxygen-evolving complex (OEC) and associated extrinsic polypeptides by hydroxylamine (HA) washing abolished the slow binding kinetics as well as the destabilizing effects on the charge-separated state. The S_2-multiline electron paramagnetic resonance (EPR) signal and the 'split' EPR signal, originating from the $S_2Y_Z^·$ state showed no significant changes upon binding of phenolic inhibitors at the Q_B site. We thus propose a working model where Q_A redox potential is lowered by short-range conformational changes induced by phenolic inhibitor binding at the Q_B niche. Long-range effects of HA-washing eliminate this interaction, possibly by allowing more flexibility in the Q_B site.
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1. Introduction

The photosystem (PS) II reaction center catalyzes the light-driven transfer of electrons from water to plastoquinone (PQ) [1–4]. Light is absorbed by the light-harvesting pigments surrounding the reaction center and transferred to a special chlorophyll species in the reaction center, known as P680, forming P680*. Initial charge separation occurs when P680* reduces a nearby pheophytin (pheo), probably through an accessory chlorophyll, forming the $P680^+pheo^-$ state. The $pheo^-$, in turn, reduces a bound PQ, known as Q_A, while $P680^+$ oxidizes a nearby redox-active tyrosine (Y_Z) to form the charge-separated state $Y_Z^·Q_A^-$. A tetranuclear manganese (Mn) cluster near Y_Z is oxidized by the tyrosine in a series of oxidation states or S-states, which are coupled to the oxidation of water to O_2 [5–8]. The electron remaining on Q_A is transferred to another plastoquinone (Q_B) that is loosely bound to the Q_B niche, forming the plastosemiquinone, Q_B^-. After another successive electron transfer from Q_A, Q_B^- is reduced to a plastoquinol with the concomitant uptake of 2 H^+ and is exchanged for PQ from the plastoquinone pool (for reviews, see Refs. [4,9]).

Electron transfer from Q_A to Q_B is inhibited by a wide variety of plastoquinone (PQ) analogs that compete with PQ at the Q_B site [10–13]. The most widely studied classes of inhibitors are the urea and triazine herbicides, such as 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and atrazine (see reviews in Refs. [14–17]). Although their binding domains are likely to overlap with the urea/triazine herbicides on the Q_B site, 'phenolic' inhibitors, which include bromoxynil, ioxynil, dinoseb, and 2,4,6-trinitrophenol (TNP) appear to inhibit in a more complex fashion (for review, see Refs. [12,14,16,18]). These compounds affect PS

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II charge recombination kinetics [12,19], the temperatures of the thermoluminescence (TL) bands [20–22], as well as the susceptibility of the reaction centers to photoinhibition [23,24]. The binding of phenolic inhibitors to the Q_B site are considerably slower than the urea/triazine herbicides [18,19,25–28], suggesting that either the binding mechanism is distinct, or that binding involves the formation of an energetically unfavorable intermediate state. In addition, phenolics can act as ADRY (acceleration of the deactivation reactions of the water-splitting enzyme Y) reagents by reducing donor-side components, though at concentrations considerably higher than those required to inhibit Q_A to Q_B electron transfer [12,29–31].

In this work, we present a detailed study of the inhibition of the Q_B site by phenolic inhibitors. We provide evidence in support of a lowering of the Q_A/Q_A^- redox potential by these inhibitors as suggested previously in Ref. [24]. We also show that removal of the oxygen-evolving complex (OEC) by hydroxylamine (HA) washing leads to dramatic changes in the inhibitory properties of these inhibitors, implying long-range conformational changes between the donor-side and the Q_B binding niche. A working model incorporating both of these observations is discussed.

2. Materials and methods

2.1. Thylakoid preparation

Thylakoids were prepared from greenhouse-grown or market spinach as previously described in Ref. [32] and stored at -80 °C in 'resuspension buffer' (50 mM HEPES pH 7.6, 400 mM sucrose, 10 mM NaCl and 5 mM $MgCl_2$) at 2 mg ml^{-1} chlorophyll in the presence of 5–7% dimethylsulfoxide as a cryoprotectant.

2.2. Removal of the OEC and extrinsic polypeptides

The OEC and its extrinsic polypeptides were removed by HA washing as described in Ref. [33]. Thylakoids (2 mg ml^{-1} chlorophyll) were incubated for 10–20 min in 5 mM HA on ice and were then washed four times in the absence of HA, followed by resuspension in fresh resuspension buffer. Residual oxygen evolution rates and TL 'Q' band amplitudes in treated samples were less than 1% that of untreated samples, indicating the near-complete removal of the OEC.

2.3. Flash-induced chlorophyll fluorescence changes

Flash-induced changes in chlorophyll *a* fluorescence yield were measured using a microsecond time-resolution pulse-probe kinetic fluorimeter, based on designs previously described in Refs. [34–36]. Thylakoid samples were diluted to a chlorophyll concentration of 5 μg ml^{-1} in

resuspension buffer containing 5 μg ml^{-1} of the uncoupler, gramicidin, and 10 μM *p*-benzoquinone (*p*-BQ) added to ensure full oxidation of Q_A and Q_B in dark-adapted samples. Samples were dark-adapted for a minimum of 5 min, followed by either a single or multiple flashes. The actinic flashes were supplied by a pulsed xenon flash lamp with 4 J total energy per flash and a discharge $t_{1/2}$ of ~ 3 μs. Based on the number of flashes required to fully reduce Q_A in the presence of DCMU as described in Ref. [34], we judged the actinic flashes to be approximately 95% saturating. Each actinic flash was followed by a series of probing flashes as provided by a bank of seven light-emitting diodes (HLMP 8103 Hewlett-Packard) with a pulse half-width of 2 μs and a peak emission at 640 nm. This light was filtered with a wide-band interference filter (635DF30 Omega, Brattleboro, VT) to remove the infrared emission. Each measuring pulse excited approximately 0.1% of the PS II reaction centers per pulse. A photodiode detector-glass color filter (RG695, Schott) combination was used to measure fluorescence yield at wavelengths above about 695 nm.

2.4. Thermoluminescence

Thermoluminescence experiments were performed using the instrument and the methods described in Ref. [37]. In these experiments, the sample temperatures were ramped at 1 °C s^{-1}, while monitoring the chlorophyll luminescence. The so-called Q-band arising from charge recombination of the $S_2Q_A^-$ state was monitored after flashing 5 min dark-adapted thylakoids (1–2 mg chlorophyll ml^{-1}) at 0 °C and rapidly cooling to -30 °C. The A_T band, most likely arising from the recombination of $Y_Z^+Q_A^-$ (see discussion in Refs. [38,39]) was observed in HA-washed thylakoids after exposing the thylakoids to continuous light at -20 °C for 30 s and rapidly cooling to -50 °C.

2.5. Preparation of BBY particles

Photosystem-II-enriched (BBY) particles were prepared as essentially described in Ref. [40] and based on methods described in Refs. [41,42], to a concentration of 10–20 mg ml^{-1} chlorophyll. Oxygen evolution of the BBY particles was determined using a Clark-type oxygen electrode as previously described in Ref. [40] with rates of oxygen evolution of 400–500 μmol of O_2 (mg of chlorophyll·h)$^{-1}$ with phenyl-*p*-benzoquinone (PPBQ) as an electron acceptor. Assuming 250 chlorophyll per reaction center as in Ref. [43] and using the method of Arnon [44] to measure the chlorophyll concentration, we approximated the concentration of PS II reaction centers, which varied between 50 and 100 μM, depending on the batch. PS II samples were trapped in the $S_2Q_A^-$ state by illumination for 1 min at 0 °C (ice) or for 5 min at 195 K (dry ice/methanol) as described in Ref. [40]. PS II samples were trapped in the S_2Q_A state by illumination for 1 min at 195

K and then annealed at 0 °C (ice) for 30 s in the presence of 1 mM ferricyanide.

2.6. Acetate-treated BBY particles

BBY particles were acetate-treated as described in Refs. [45–47]. Samples were washed twice with 'SME' buffer (i.e. 400 mM sucrose; 50 mM MES-NaOH, 1 mM EDTA, pH 5.5) and once with 'SMAcE' buffer (i.e. 400 mM sucrose; 50 mM MES-NaOH; 0.5 M sodium acetate; 1 mM EDTA, pH 5.5). Samples were incubated with either 1 mM PPBQ or 1 mM ferricyanide in the presence or absence of Q_B site inhibitors. After 5 s illumination of these samples at 4 °C, the $S_2Y_{\dot{Z}}Q_A^-$ state is formed, giving rise to the so-called 'split' electron paramagnetic resonance (EPR) signal [46,48,49].

2.7. EPR spectroscopy

EPR spectra were collected at 7 K with a Bruker ECS106 X-band continuous wave (CW)-EPR spectrometer (Bruker Instruments, Billerica, MA) equipped with an Oxford ESR900 liquid helium cryostat and an ITC503 temperature controller (Oxford Instruments, Oxford, UK). EPR parameters are described in the figure legends.

3. Results

3.1. Effects of phenolic inhibitors on intact PS II reaction centers

Fig. 1A shows the rate of Q_A oxidation after a flash, monitored by chlorophyll fluorescence decay kinetics. The $t_{1/2}$ of fluorescence decay increased from about 500 μs in uninhibited samples [50–52] to ~ 300 ms in the presence of TNP (Fig. 1A) and the other phenolic inhibitors, bromoxynil and dinoseb (data not shown). Blockage with DCMU (Fig. 1A) or atrazine (data not shown) led to fluorescence decay $t_{1/2}$ of 2–3 s as previously observed [53]. Both TNP-inhibited samples and DCMU-inhibited samples showed a fluorescence rise phase in the tens of microseconds range, most likely reflecting S-state turnover [54].

In the presence of saturating concentrations between greater than 1 μM and up to 500 μM of TNP or other phenolic inhibitors, a small fraction (5–10%) of the flash-induced fluorescence decay curve remained rapid (Fig. 1A). This fast-decay phase was absent in the DCMU-inhibited samples (Fig. 1A). Within a minute of adding 1 μM of DCMU to thylakoids pre-incubated with 10 μM TNP, this fast-decay phase was replaced by a slower-decay phase, consistent with $S_2Q_A^-$ recombination in DCMU-inhibited centers. There is probably a fraction of reaction centers in this milieu that are not inhibited by TNP. However, a majority of PS II reaction centers were inhibited by TNP and this suggested that a fraction of PS
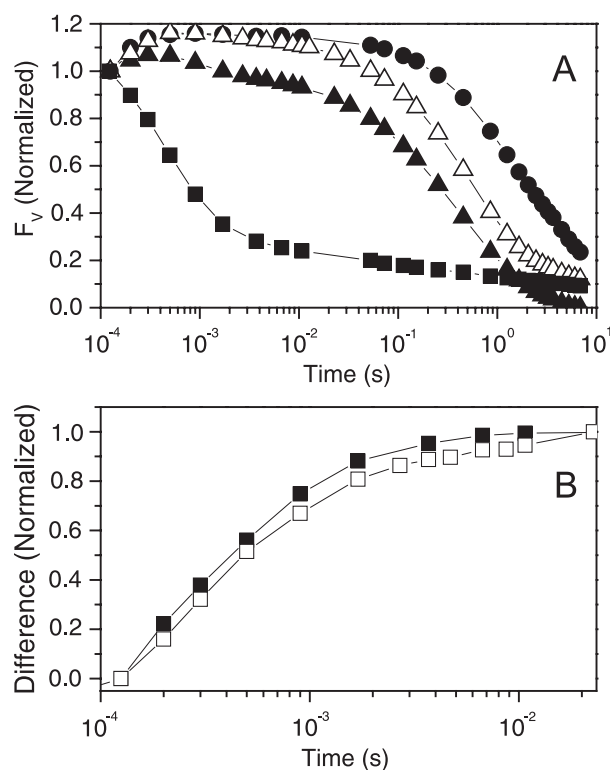

Fig. 1. Fluorescence decay kinetics of intact (i.e. not HA-washed) PS II centers. (A) Uninhibited (closed squares), 10 μM TNP-inhibited (closed triangles), 1 μM DCMU-inhibited (closed circles), and 10 μM TNP-inhibited PS II with 1 μM DCMU added rapidly (open triangles). (B) The difference between 1 μM DCMU and the uninhibited control (closed squares) and the difference between 10 μM TNP/1 μM DCMU and 10 μM TNP (open squares).

II centers were insensitive to TNP, but had high affinity for DCMU.

To characterize the Q_A to Q_B electron transfer in the TNP-insensitive PS II reaction centers, we subtracted the decay kinetics from centers inhibited with both 10 μM TNP and 1 μM DCMU from those inhibited with only 10 μM TNP (see Fig. 1B). The initial rise in fluorescence following a flash in these samples was virtually identical, allowing us to eliminate interference from the overlapping tens of μs rise phase, resulting from slower phases of $P680^+$ reduction [34,54,55]. Therefore, the early part of the difference kinetics should reflect the fluorescence decay associated with Q_A to Q_B electron transfer in the presence of TNP. The decay $t_{1/2}$ for the difference kinetics in the 0–10 ms time domain, estimated in this way, was on the order of 1 ms, i.e. significantly slower than the predominant Q_A to Q_B electron transfer observed in uninhibited samples (Fig. 1B, closed squares). It is not clear that these insensitive centers exhibit nonlinear fluorescence like the bulk of the reaction centers, but if they did, then our measurements underestimate the true $t_{1/2}$ since it was determined under conditions of high overall fluorescence yield [56]. This suggests that the insensitive centers exhibit modified Q_A to Q_B electron transfer kinetics. We were unable to determine the K_I for TNP in these insensitive

centers since inhibition of these centers by TNP occurs at a concentration where the ADRY properties of this inhibitor were noticeable.

3.2. Effects of phenolic inhibitors on the $Y_Z^{\cdot}Q_A^-$ recombination

Fig. 2 shows the decay of flash-induced fluorescence decay in HA-washed thylakoids. In the absence of Q_B site inhibitors, HA-washing resulted in a slowing of Q_A to Q_B electron transfer to a $t_{1/2}$ of ~ 2 ms, as previously observed [57–59]. There was also a large increase in the slower fluorescence decay phases, possibly reflecting changes in the Q_B site [59,60]. All of the phenolic inhibitors tested (i.e. bromoxynil, dinoseb, and TNP), as well as DCMU, effectively blocked Q_A to Q_B electron transfer in HA-washed reaction centers, as demonstrated by a slowing in the predominant decay phase to about 200 ms. Very long decay phases also appeared, which we attribute to a combination of small actinic effects of the measuring beam and the trapping of electrons on Q_A upon reduction of oxidized donor-side components by TNP. However, we expect the contribution from the reduction of oxidized donor-side components to be small, since there is no significant decrease in the A_T band thermoluminescence at a similar concentration of inhibitor (see below).

3.3. Effect of phenolic inhibitors on the A_T thermoluminescence bands

The temperatures of the so-called A_T thermoluminescence (TL) band of HA-washed thylakoids (Fig. 3), which likely reflects $Y_Z^{\cdot}Q_A^-$ recombination [33,38,39 and references therein] were unaffected by treatment with DCMU, TNP, bromoxynil, or dinoseb, showing a TL band at − 14 °C under our conditions. We note that our TL data are in
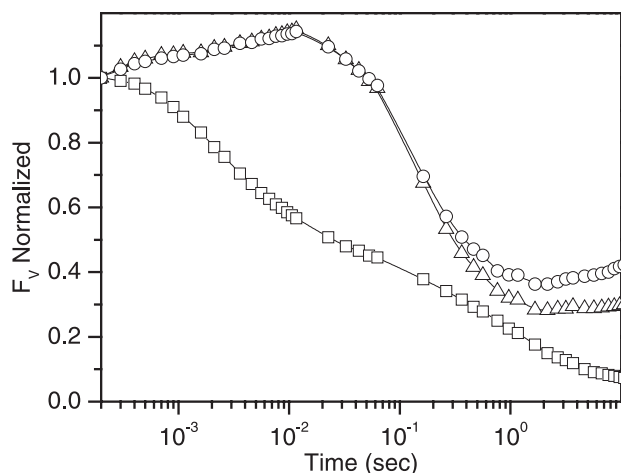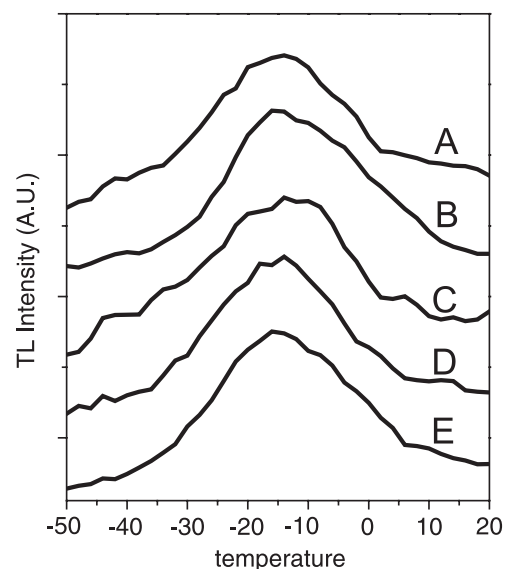

Fig. 3. A_T thermoluminescence bands of HA-washed thylakoids (A) without inhibitors, or inhibited with (B) 3 μM dinoseb, (C) 3 μM bromoxynil, (D) 3 μM TNP, or (E) 3 μM DCMU. Thermoluminescence parameters are described in the text.

contradiction with that reported by Krieger-Liszkay and Rutherford [24], who found dramatic differences between bromoxynil and DCMU-induced TL bands. We suggest that the TL data obtained by these authors may be skewed because of the ADRY effects induced by the high concentrations (100 μM) of bromoxynil used. In our hands, these concentrations of bromoxynil, TNP, and dinoseb completely abolished the A_T bands (data not shown), probably because of their ADRY properties ([27,61]; A.G. Roberts, D.M. Kramer, unpublished results).

3.4. Thermodynamics of phenolic inhibitor binding

Using the fluorescence yield taken 10 ms after flash excitation as an indicator of phenolic herbicide binding to the Q_B site (Fig. 4A), we obtained a K_I for TNP of about 375 nM. This is consistent with the binding affinities obtained previously for TNP and the other phenolic inhibitors [12,18,26,62]. Removal of the OEC by HA treatment significantly decreased the K_I values of phenolic inhibitors to about ~ 70 nM, closer to DCMU in intact and HA-treated PS II reaction centers (i.e. $K_I = 50–70$ nM [19], and data not shown).

The binding kinetics for TNP (Fig. 4B) and other phenolic inhibitors (data not shown) to intact PS II were found to be unusually slow with a $t_{1/2}$ for blockage of ~ 78 s upon the addition of 0.5 μM TNP [12,18]. The $t_{1/2}$ for blockage was inversely proportional to the concentrations of TNP added (data not shown), allowing us to calculate a second-order rate constant for binding (k_{on}) of 26 mM^{-1} s^{-1}. This is compared to a k_{on} for the triazine/urea herbicides of ~ 1000 mM^{-1} s^{-1} [25]. It may be concluded that a relatively slow k_{on} is a general feature of phenolic

Fig. 2. Fluorescence decay kinetics of HA-washed PS II, lacking an OEC with uninhibited (squares), 1 μM TNP-inhibited (triangles), and 1 μM DCMU-inhibited PS II (circles).
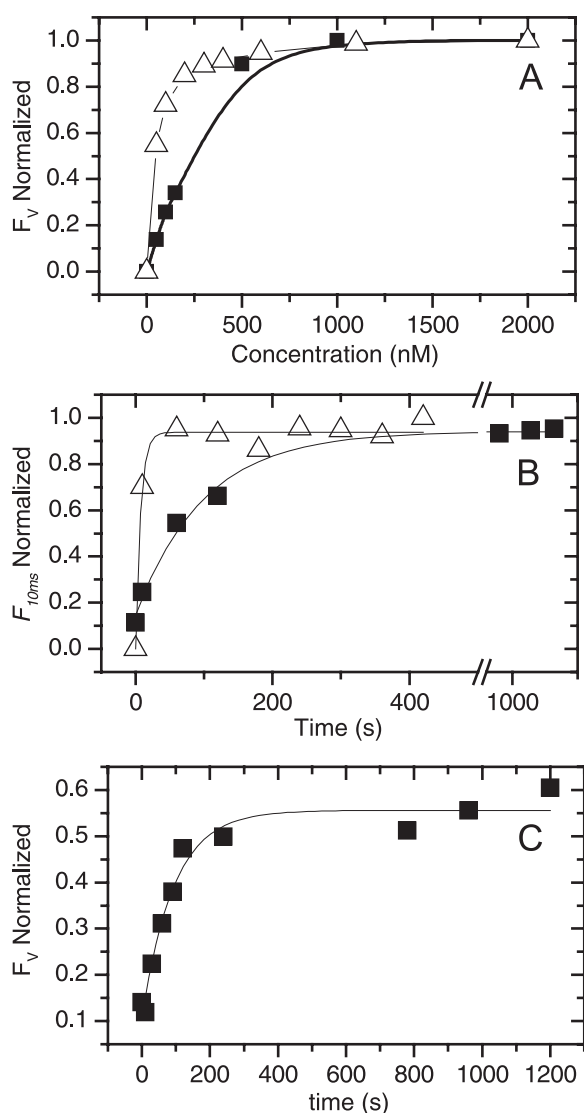
Fig. 4. Normalized fluorescence yield of thylakoids inhibited with TNP. (A) Normalized fluorescence yield as a function of TNP concentration. (B) Normalized fluorescence yield at 10 ms ($F_{10\ ms}$) after the addition of 0.5 μM TNP to uninhibited samples. (C) Normalized fluorescence yield after the addition of 10 μM DCMU in thylakoids inhibited with 1 μM TNP. The measurement of fluorescence yield was taken 2 s after a saturating actinic flash. Closed boxes are intact PS II reaction centers and open triangles are HA-treated PS II.

inhibitors [18,26,63]. After HA washing, the rate of binding was dramatically accelerated to a k_{on} of ~ 200 mM^{-1} s^{-1} (Fig. 4B, open triangles).

Addition of excess DCMU (10 μM) to samples previously inhibited with 1 μM TNP (Fig. 4C) led to a gradual increase in the $t_{1/2}$ for recombination from ~ 300 ms to 2–3 s from kinetics characteristic of TNP-blocked centers to those characteristic of DCMU-blocked centers [25,53,64]. This was consistent with overlapping binding sites for the two types of inhibitors, as previously discussed [27]. Since the binding of DCMU in the absence of TNP is rapid, the rate-limiting step for displacement is expected to be the unbind-

ing of TNP. The increase in decay $t_{1/2}$ indicated by the fluorescence yield at 2 s after the flash was used to estimate the first-order rate constant for unbinding (k_{off}) of TNP of 0.013 s^{-1}. The values of k_{on} and k_{off} determined from the above kinetics assays were used to calculate a K_I of about 500 nM, which is in reasonable agreement with that determined by the equilibrium titration (i.e. $K_I = \sim 375$ nM).

3.5. The effect of $S_2Q_A^-$ on the binding of TNP

Since the $S_2Q_A^-$ state appears to be higher in energy in the presence of the phenolic inhibitors than with DCMU, it follows that the formation of this state should affect inhibitor binding. The following experiments were designed to test this (Fig. 5A–D). Fig. 5A shows that a single actinic flash given to TNP-inhibited thylakoids resulted in a chlorophyll fluorescence with a $t_{1/2}$ of about 300 ms. Since the flash excitation is short (~ 3 μs) compared to the k_{off} for TNP, the 300-ms decay kinetics should predominantly reflect PS II reaction centers inhibited by TNP in the S_1Q_A state. When a second actinic flash was given 6 s after the first, a significant fraction of the slow phase was replaced with a rapid (< 1 ms) decay phase, similar to that observed in uninhibited samples. Subsequent flashes (at 0.167 Hz) further decreased the slow phase while increasing the rapid decay phase. After several flashes, the overall fluorescence decay kinetics closely resembled those of uninhibited samples [50–52]. Since the k_{on} for PQ is much more rapid than that for TNP (see above), PQ could then bind to the Q_B site, allowing normal Q_A to Q_B electron transfer to proceed on subsequent flashes. This is expected to have resulted in the formation and accumulation of the $S_2Q_B^-$ state, which decays in approximately 22 s [65].

We next followed the time course for rebinding of TNP following multiple-flash excitation by measuring flash-induced fluorescence kinetics at variable times after the flash train. The second-order decay constant for recovery of the fluorescence decay kinetics to return to the fully dark-adapted condition, reflecting the TNP-inhibited PS II in the S_1Q_A state, was approximately 25 mM^{-1} s^{-1} (data not shown), which was similar to the apparent k_{on} of TNP ($k_{on} = 26$ mM^{-1} s^{-1}). This implies that the rate-limiting step for recovery of the fluorescence decay kinetics was TNP rebinding at the Q_B site as observed previously for other phenolic herbicides [19].

We thus argue that the normalized chlorophyll fluorescence yield 10 ms after a saturating actinic flash, a parameter we term, $F_{10\ ms}$, should reflect the fraction of Q_B sites blocked with inhibitors. It is important to keep in mind that the relationship between fluorescence yield and the concentration of PS II centers in high fluorescence states is nonlinear, and thus the $F_{10\ ms}$ will not be a linear indicator of the number of blocked Q_B sites. However, the effect is relatively small and when corrections were made for this nonlinearity using a reasonable antenna model [66,67], our results were within 10–25% of that arrived at using uncorrected fluorescence values [68,69]. Since correction of the
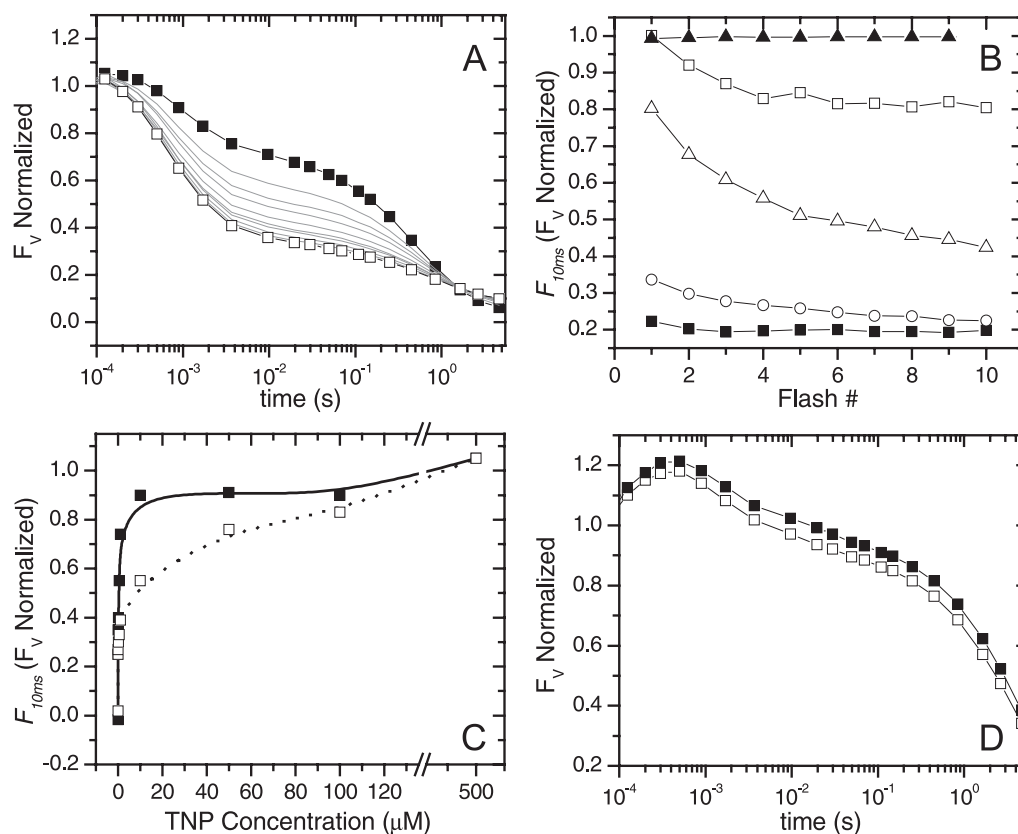
Fig. 5. Effects of multiple excitation flashes on chlorophyll fluorescence decay kinetics. Saturating single-turnover flashes were given at 0.167 Hz. (A) Fluorescence decay kinetics after a single flash (closed squares), after 2–9 flashes (intermediate dotted traces) and after 10 flashes with 500 nM TNP (open squares). (B) Normalized fluorescence yield 10 ms after flash excitation as a function of flash number in uninhibited controls (closed squares), 0.1 μM TNP (open circles), 1 μM TNP (open triangles), 10 μM TNP (open squares), and 10 μM DCMU (closed triangles). (C) Normalized fluorescence yield 10 ms after flash excitation ($F_{10\ ms}$) as a function TNP concentration with no preflashing (closed squares) and after 9 preflashes (open squares). (D) Fluorescence decay kinetics of HA-washed PS II after a (closed squares) single flash and (open squares) after 10 flashes with 500 nM TNP.

fluorescence nonlinearity requires assumptions about antenna function [66,67], we prefer to present the data in its uncorrected form.

Fig. 5B shows the effects of flash excitation (at 0.167 Hz) on $F_{10\ ms}$. The fluorescence yield in this figure was normalized against the first time point (65 μs after the flash) to account for small changes in maximal fluorescence yield that occurred over time. In DCMU-treated thylakoids, $F_{10\ ms}$ remained relatively constant for each of the 10 flashes, but significantly higher than the control (no inhibitors), indicating that blockage of Q_A reoxidation by DCMU was not affected by repeated flash excitation. In the presence of 0.1 μM TNP, $F_{10\ ms}$ after the first flash was 1.5-fold higher than in the absence of inhibitors, indicating a blockage of about 20% of the Q_B sites. Successive flashes resulted in a lowering of $F_{10\ ms}$, reaching a 'steady-state' level slightly higher than control by the tenth flash. A similar behavior was observed after addition of 1 and 10 μM TNP, although the steady-state fluorescence yield levels increased with increasing TNP concentration. This trend continued at even higher concentrations of TNP (data not shown), but significant effects from the ADRY properties of TNP become evident, preventing detailed analysis of the data.

If we consider the k_{off} of TNP (0.013 s^{-1}, see above) in PS II reaction centers with subsaturating TNP, we should only expect roughly ~1% decrease in the $F_{10\ ms}$ as a result of inhibitor displacement by Q_B^- after flash excitation; instead, we see a considerably greater decrease in $F_{10\ ms}$ per flash. We interpret the flash-induced lowering of $F_{10\ ms}$ as reflecting a change in the binding properties of the Q_B site upon formation of the $S_2Q_A^-$ state, resulting in the displacement of TNP from the site. Subsequent binding of PQ to the Q_B site should have resulted in oxidation of Q_A and the formation of the Q_B^- state, which displays low fluorescence.

From the above, we argue that $F_{10\ ms}$ after the first flash should reflect the K_I for TNP when PS II centers are in the S_1Q_A state, while in the 'steady-state' (after 10 flashes), should reflect the binding of TNP to the Q_B site in the $S_2Q_A^-$ state as well as the equilibrium for electron sharing between the Q_A and Q_B semiquinones, when an unpaired electron is present on the acceptor complex [10,12]. Fig. 5C is a plot of $F_{10\ ms}$ after the first and tenth flashes as a function of TNP concentration. The apparent K_I for phenolic inhibitors blocking at the Q_B site after the first flash (i.e. in the S_1Q_A state) was roughly 300 nM, which is similar to that found previously [19].

After 10 flashes, $F_{10\ ms}$ showed two distinct concentration-dependent phases, as reflected in a second-order exponential curve fit in Fig. 5C. The sensitive phase had an apparent K_I of about 300 nM, similar to the K_I of TNP in dark-adapted PS II. This was followed by a less-sensitive phase that did not saturate over the concentration range that was used. However, this phase did fit well to a K_I of about 30 μM, 100-fold weaker than in the case of dark-adapted PS II. These results can be interpreted within the context of the two-electron gate mechanism. As discussed above, the formation of the $S_2Q_A^-$ state is expected to displace TNP from a fraction of centers, allowing them to turn over normally with subsequent flashes. After multiple turnovers, the redox state of the acceptor complex in these uninhibited centers will become scrambled, i.e. about half of the acceptor complexes will contain either a Q_A^- or Q_B^- while others will be fully oxidized. The latter (oxidized) reaction centers should rebind TNP with a K_D similar to that observed in nonflashed samples. We thus assign the sensitive TNP titration phase to such centers. In centers with Q_B^-, TNP binding would require oxidation of Q_B by Q_A, followed by release of Q_B from its binding site. Thus, we would expect a ~ 10-fold increase in the K_D solely as a result of electron sharing between Q_A and Q_B at the assay pH [10,12]. The best-fit titration curve showed a 100-fold decrease in binding and suggest that TNP affinity to the open Q_B site was decreased by about 10-fold after formation of the $S_2Q_A^-$ state (see Discussion).

Fig. 5D shows that in HA-washed samples, multiple-flash excitation had little effect on fluorescence decay in the presence of 500 nM TNP or DCMU. If we assume an equilibrium constant between Q_A and Q_B^- of 10, then the K_I for binding in HA-treated PS II with one electron present (i.e. $S_2Q_AQ_B^-$) should be 700 nM, so that a significant displacement is still expected even at 500 nM. Similar results were found in the presence of dinoseb and bromoxynil (data not shown). These results persisted even when flashes were given at >100 Hz (data not shown) or where lower concentrations of phenolics were used (data not shown). Thus, although phenolic inhibitors affected the stability of the $S_2Q_A^-$ state and vice versa, they did not appear to affect the stability of the $Y_Z^·Q_A^-$ state.

3.6. Effects of phenolic inhibitors on the S_2-multiline EPR signal

Fig. 6A and B show the S_2-multiline (light minus dark) with the overlapping $Fe^{2+}Q_A^-$ signal ($g = 1.90$ to $g = 1.67$ region) of BBY particles that were inhibited with TNP and DCMU (for reviews, see Refs. [6,70–72]). There were no apparent differences in the position or line shape of the S_2-multiline between these control and TNP-treated samples. Neither addition of dinoseb nor bromoxynil affected the spectrum (data not shown). Some samples inhibited with phenolic inhibitors showed decreases in the amplitude (~ 30%) of the S_2-multiline and $Y_D^·$ radical signal ($g =$

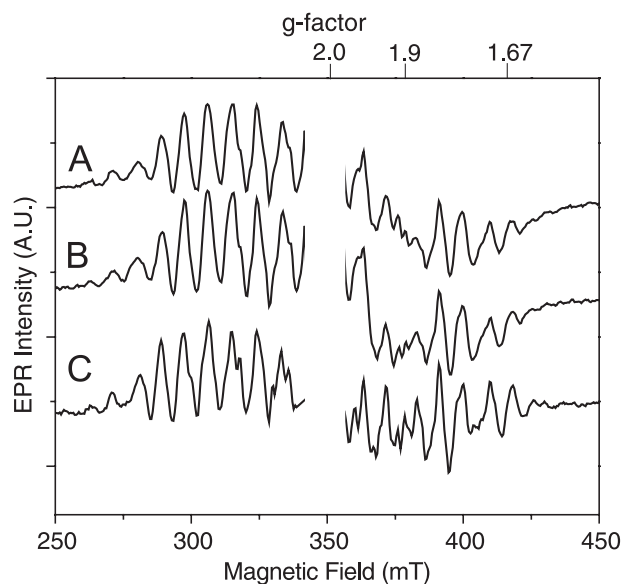Fig. 6. S_2-multiline EPR signal in BBY particles with ~ 50 μM of PS II reaction centers treated with (A) 50 μM DCMU, (B) 50 μM TNP, (C) 50 μM TNP and 1 mM ferricyanide. The amplitudes of the EPR signals were normalized to the $Y_D^·$ radical signal. EPR parameters were: microwave power, 5 mW; modulation amplitude, 10 G; conversion time, 82 ms; time constant, 41 ms.

~ 2.0) that we attribute to the ADRY properties of these inhibitors [57,61]. The BBY particles in these experiments were in the $S_2Q_A^-$ state, where binding of TNP or other phenolic inhibitors will be weak and might not induce strong effects on the S_2-multiline. Therefore, we repeated these experiments in the S_2Q_A state by oxidizing Q_A^- with the oxidized nonheme iron (for reviews, see Refs. [73,74]). Samples were illuminated at 195 K in the presence of 1 mM ferricyanide, leading to a single charge separation [75] and the formation of Q_A^-. This was annealed for 30 s at 0 °C (ice) in the dark, which allowed oxidation of the Q_A^- by the nonheme iron on a microsecond time scale [73,74]. The S_2-multiline spectra from BBY particles treated with TNP (Fig. 6C) had only a small $Q_A^-Fe^{2+}$ signal [72,76], which we attribute to multiple turnovers in a fraction of PS II reaction centers [75]. The S_2-multiline resulting from the TNP treatment (Fig. 6C) showed no noticeable differences in line shape or position, when compared to treatment with any of the other inhibitors (i.e. DCMU, bromoxynil, and dinoseb). We conclude that phenolic inhibitors do not significantly distort the Mn-cluster in the presence of S_2Q_A and the $S_2Q_A^-$ states, and that the redox state of Q_A (i.e. Q_A or Q_A^-) does not significantly distort the Mn-cluster.

3.7. Effects of phenolics on the 'split' EPR signal

Fig. 7 shows the 'split' EPR signal from acetate-treated BBY particles, resulting in the $S_2Y_Z^·$ state [46,48,49]. The line shape and position of the 'split' EPR signal was virtually identical in all the samples, including TNP and the other phenolics (data not shown). In some of the samples
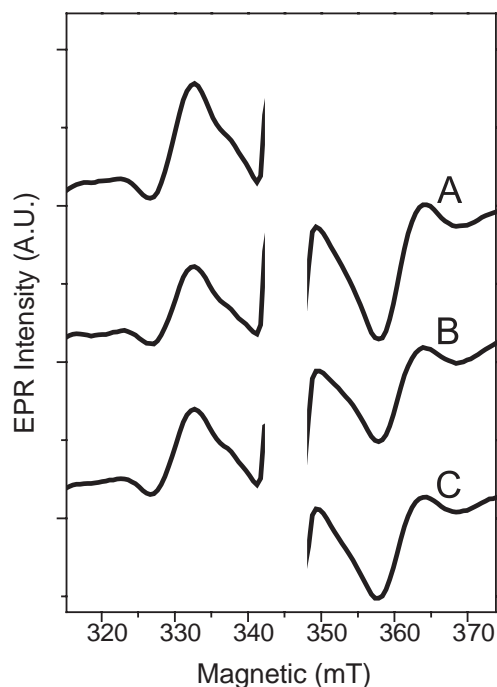
Fig. 7. EPR of acetate-treated BBY particles with ~ 100 μM of PS II reaction centers with (A) 1 mM PPBQ, (B) 1 mM PPBQ and DCMU, (C) 1 mM PPBQ and TNP. The figure was normalized and the EPR parameters were the same as in Fig. 6.

that were treated with phenolic inhibitors, there was decrease in the amplitude of the 'split' and the $Y_D^.$ EPR signal, which we attribute to the ADRY properties of these inhibitors. In acetate-treated PS II reaction centers, we conclude that there were no apparent distortional effects of phenolic inhibitors to the Mn cluster, Y_Z, or their magnetic interaction by phenolic inhibitors binding at the Q_B site.

4. Discussion

Compared with the well-characterized inhibitory action of urea/triazine herbicides, the inhibition of PS II by phenolic inhibitors is complex [12,14,16,27,29,77]. Of particular interest are the effects of these inhibitors on the energetics of PS II electron transfer [10,14,20,78], potentially making these inhibitors useful tools for the study of the relationship between PS II energetics and function. The study presented here allows us to make several conclusions about PS II, and its interaction with this class of inhibitors.

4.1. Binding properties of phenolic inhibitors reveals PS II heterogeneity

A population of PS II reaction centers (5–10%) exhibits modified Q_A to Q_B electron transfer in the presence of saturating concentrations of TNP and the other phenolic inhibitors. The inhibitor insensitivity may be the result of

differences in the conformation of the Q_B binding niche, including potential differences in the subunit composition of the donor side in these reaction centers. The precise origin of the effect is unclear, but inhomogeneity in PS II reaction centers has been related to a variety of phenomena, including the rate of turnover of the D1 protein, regulation of phosphorylation, and location within the thylakoid membrane (see review [79]).

4.2. The effects of phenolic inhibitors on PS II energetics

The ~ 10-fold-more rapid $S_2Q_A^-$ back-reaction kinetics as compared to PS II reaction centers inhibited by DCMU suggests that the $S_2Q_A^-$ state is 10-fold destabilized by this inhibitor (Fig. 1A). In the expected complementary fashion, the affinity of phenolic herbicides to the Q_B site decreased 10-fold in the presence of the $S_2Q_A^-$ state induced by multiple flashing (Fig. 5C). In principle, these effects may be explained by changes in either the S_1/S_2 or the Q_A/Q_A^- redox couples. Because of the proximity of the Q_A and the Q_B binding niches (i.e. ~ 20 Å [2]) and the low pK_a of phenolic inhibitors (e.g. $pK_a = 0.38$–4.1, [80–82]), the most obvious explanation is that the redox properties of Q_A are affected by the electrostatic interaction with the charge of the bound phenolic anion, destabilizing the Q_A/Q_A^- redox couple (but see below). Indeed, redox titrations by Krieger-Liszkay and Rutherford [24] showed a significant decrease in the midpoint potential of the Q_A/Q_A^- redox couple in the presence of the phenolic inhibitor, bromoxynil. It should be noted, however, that to avoid possible interference, these titrations were carried out in the absence of redox mediators [24,83], and it is unclear whether equilibration of the ambient potential between the electrode and Q_A was reached.

Our data shows that when the OEC and the extrinsics were removed by HA treatment, the phenolic inhibitors no longer destabilized the charge-separated state with respect to samples treated with DCMU, as shown by their similar fluorescence decay kinetics and A_T thermoluminescence bands (Figs. 2 and 3). Furthermore, we found that the removal of the OEC by HA-washing dramatically increased the k_{on} and binding affinity of phenolic inhibitors (Fig. 4A,B). Because of the distance between the Q_B site and the OEC (i.e. >40 Å [2,84]), we argue that any mutual effects between Q_B site binding and Q_A redox potential or state would require long-range conformational changes. Evidence of long-range interactions between the donor and the acceptor sides has been observed previously in Ca^{2+}-depleted PS II [85], donor-side mutations of the D1 protein [86], and acceptor side mutations in *Synechocystis* PCC 6714 [87]. In addition, removal of the extrinsic polypeptides of the OEC was shown by electron microscopy to have dramatic effects on the conformation of the PS II supercomplex [88]. Alternatively, phenolic inhibitors at the Q_B site could modulate the redox properties of the S_1/S_2 redox couple by long-range conformational changes. To probe for such

effects, we measured the EPR spectrum of the S_2-state of the Mn cluster, which is known to be sensitive to certain treatments that are believed to affect the geometry of the cluster (reviewed in Ref. [6]). We found no such changes in either the S_2-multiline or the so-called 'split' EPR signal (Figs. 6 and 7). It is possible that the redox state of the S_1/S_2 redox couple could be affected without inducing noticeable changes in the S_2-state EPR signals. This could include changes in the S_1-state EPR signals, as was seen after the removal of the 17 and 23 kDa proteins [40]. However, combined with the redox titrations of Q_A in the presence of phenolic inhibitors [24], the data are more consistent with effects on the Q_A/Q_A^- redox potential. Since the $S_2Q_A^-$ back-reaction rate is increased $\sim$ 10-fold in the presence of phenolic inhibitors and the affinity of these herbicides is decreased $\sim$ 10-fold in the presence of the $S_2Q_A^-$ state, it would suggest that phenolic inhibitor binding at the Q_B site lowers the Q_A/Q_A^- redox couple by 10-fold or $\sim$ 60 mV as previously suggested in Ref. [24].

In principle, the influence of phenolic inhibitors on the Q_A redox potential could arise from purely electrostatic interactions, and/or from binding-induced conformational changes, which could alter the properties of Q_A. We suggest that HA treatment allows greater flexibility in the Q_B pocket, allowing the phenolic inhibitor to bind without transmitting conformational changes to the Q_A site. The increased k_{on} combined with tighter binding after HA washing is consistent with this hypothesis and we suggest it as a working model.

Acknowledgements

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