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Journal

The Journal of Physical Chemistry Letters, 8(22)

ISSN
1948-7185

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Publication Date

2017-11-16

DOI
10.1021/acs.jpclett.7b02486

Peer reviewed

THE JOURNAL OF PHYSICAL CHEMISTRY Letters

Letter

Cite This: J. Phys. Chem. Lett. 2017, 8, 5548-5554

pubs.acs.org/JPCL

Snapshot Transient Absorption Spectroscopy of Carotenoid Radical Cations in High-Light-Acclimating Thylakoid Membranes

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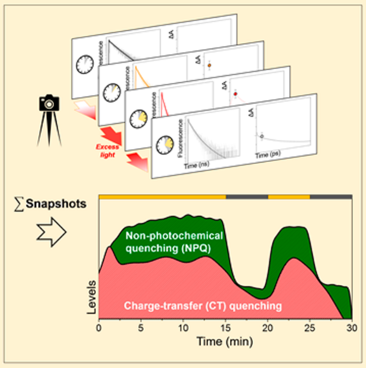
Supporting Information

ABSTRACT: Nonphotochemical quenching mechanisms regulate light harvesting in oxygenic photosynthesis. Measurement techniques for nonphotochemical quenching have typically focused on downstream effects of quenching, such as measuring reduced chlorophyll fluorescence. Here, to directly measure a species involved in quenching, we report snapshot transient absorption (TA) spectroscopy, which rapidly tracks carotenoid radical cation signals as samples acclimate to excess light. The formation of zeaxanthin radical cations, which is possible evidence of zeaxanthin−chlorophyll charge-transfer (CT) quenching, was investigated in spinach thylakoids. Together with fluorescence lifetime snapshot data and time-resolved high-performance liquid chromatography (HPLC) measurements, snapshot TA reveals that $Zea^{\bullet+}$ formation is closely related to energy-dependent quenching (qE) in nonphotochemical quenching. Quantitative and dynamic information on CT quenching discussed in this work give insight into the design principles of photoprotection in natural photosynthesis.



Nonphotochemical quenching (NPQ) describes a collection of mechanisms that photosynthetic organisms use to control the amount of excitation energy reaching reaction centers and to minimize potential oxidative damage. These mechanisms have typically been divided into groups based on their time scales of activation; the fastest set are known as energy-dependent quenching (qE) mechanisms, and they turn on within seconds to minutes of initial high light exposure.[1] Other types of quenching have activation times varying from tens of minutes to hours. Though all types of quenching protect plants from photoinhibition, the rapid response of qE mechanisms makes them responsible for the majority of quenching in the early stages of light acclimation, allowing for higher seed production under fluctuating light conditions.[2] The carotenoid zeaxanthin (Zea) is required for full qE quenching, but the production of the majority of Zea requires enzymatic conversion from violaxanthin (Vio) in high light.[3−5] It is still unknown exactly how Zea participates in qE quenching, but two possible and nonmutually exclusive mechanisms have been suggested. One involves charge-transfer quenching (CT quenching), in which Zea and a neighboring chlorophyll (Chl) molecule accept excitation energy as a dimer and undergo charge separation followed by recombination, transiently forming a Zea cation and Chl anion.[6] The second mechanism involves excitation energy transfer from an excited Chl Q_y state to a Zea S_1 state, which then rapidly relaxes with a lifetime of ~9 ps.[7,8] In addition to Zea, the carotenoid lutein (Lut) is thought to be directly involved in quenching via similar mechanisms.[9,10]

In previous work, the Zea radical cation ($Zea^{\bullet+}$) was observed in high-light-acclimated plant thylakoids using transient absorption (TA) spectroscopy.[6] However, these thylakoids had been high-light-acclimated for over 30 min before measurement, which does not indicate whether CT quenching is activated within the first few minutes of high light exposure, the time scale of qE activation. $Zea^{\bullet+}$ has also been observed in isolated minor (monomeric) light-harvesting complexes containing Zea,[11,12] but these protein conditions may not be indicative of in vivo behavior, and once again, do not give information about when CT quenching turns on during light acclimation. In a recent study, Dall'Osto and coworkers concluded that the trimeric light-harvesting complex II (LHCII) is the location of a more slowly activated (several minutes) quenching mechanism that does not involve

Received: September 20, 2017
Accepted: October 30, 2017
Published: October 30, 2017

formation of Zea$^{•+}$ in vivo.[13] This implies that CT quenching may be one of multiple quenching (qE) mechanisms.

In order to obtain direct evidence on the time scale of CT quenching, we developed a technique that we call snapshot TA spectroscopy, which uses a TA setup at a fixed time delay to allow for data collection within a 10 s window, in intervals as short as a few tens of seconds. Although this method can be exploited to study the formation of other species, our work has specifically focused on the formation of Zea$^{•+}$ in thylakoid membranes to gather quantitative and dynamic information on the time dependence of Zea−Chl CT quenching. We interpret the snapshot TA data in conjunction with fluorescence lifetime snapshot and time-resolved HPLC data.

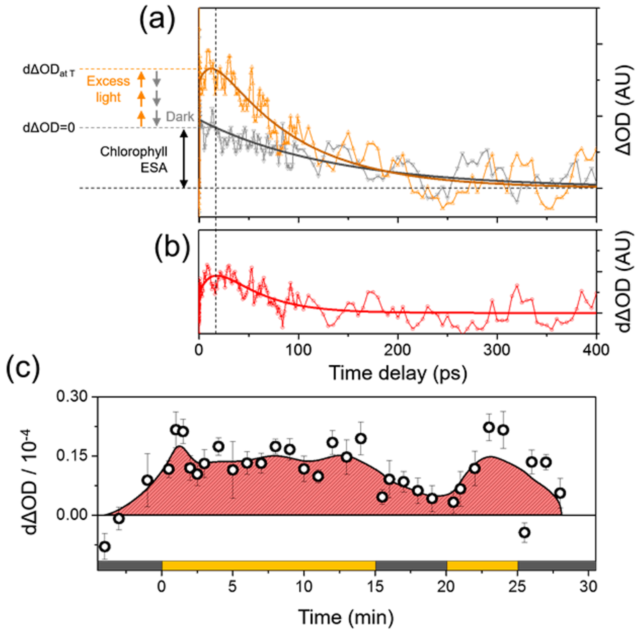


Figure 1. (a) TA kinetics for spinach thylakoid membranes under high-light-acclimated (triangles, yellow) and dark-acclimated (inverted triangles, gray) conditions. (b) Difference between light-acclimated and dark-acclimated kinetic traces. (c) Snapshot TA data obtained 20 ps after excitation with the zero-line representing the averaged signal during the initial dark period. Data are presented as the means ± SE (n = 5), and the solid line represents smoothed curves. Spinach thylakoid membranes were excited and probed at 650 and 1000 nm. The time sequence of actinic light on (yellow) and off (dark gray) is presented in the bottom bar.

Figure 1a exhibits TA kinetic profiles probed at 1000 nm for dark- and light-acclimated spinach thylakoid membranes upon excitation of the Chl Q_y band. Light acclimation of the thylakoids, induced by continuous irradiation at 850 μmol photons·m^{-2}·s^{-1}, leads to the formation of Zea$^{•+}$, resulting in additional rise (15.4 ps) and decay (40 ps) components (Figure 1b). In a previous report,[6] the TA kinetic traces of the Zea$^{•+}$-depleted *Arabidposis thaliana* (*npq4* mutant) indicated that the Chl excited-state absorption (ESA) signal is nearly identical in dark- and light-acclimated samples at 1000 nm. Therefore, Chl ESA dynamics and Chl*−Chl* annihilation were thought to contribute equally to the near-IR TA signals of dark-acclimated and high-light-acclimated thylakoids at the same Chl concentration and excitation laser intensity, and the observed difference kinetics directly indicate the population of the charge-separation states (Chl$^{•-}$ and Zea$^{•+}$).[14] The reconstructed difference spectrum in Figure S1 indicates the characteristic Zea$^{•+}$ absorption, consistent with our previous observations.[6] When following the formation of Zea$^{•+}$ in spinach thylakoids, the maximum difference in the decay traces of dark- and light-acclimated samples occurs at a time delay of 20 ps and at a detection wavelength of 1000 nm. By focusing only on that wavelength and delay time, we were able to acquire a data point every 30 s, making the snapshot TA method a valuable complement to fluorescence for tracking qE on the seconds to minutes time scale. The duration of the data acquisition window is limited by the signal-to-noise (SN) ratio. To increase the SN ratio, we placed appropriate sets of filters and polarizers, as described in the Experimental Methods section. Using dark-acclimated samples, we first established a baseline, corresponding to the ESA of Chl, and then began the light acclimation sequence (Figure 1a,c).

Figure 1c shows the difference between the TA signal from dark-acclimated sample and the signal at various times during light acclimation. There is a sharp rise in Zea$^{•+}$ absorption signal within 2 min of the first light acclimation period, supporting the idea that CT quenching is part of a qE mechanism. The signal drops and then plateaus after about 5 min of light exposure. We suggest that this is due to trimeric LHCII migration away from the photosystem II (PSII) supercomplex, which is proposed to occur within 5 min of high light exposure.[15,16] If, as suggested previously,[11,12,17] CT quenching occurs in the monomeric LHCII, this reduces the amount of excitation energy funneled to the CT quenching site. It is noteworthy that the Zea$^{•+}$ TA signal slowly decreased in dark periods despite the near-constant Zea concentration, indicative of asymmetric induction-relaxation of the CT quenching mechanism. Another possible quencher, Lut$^{•+}$, exhibits maximum peak absorption at 920 nm, which is noticeably blue-shifted relative to the spectrum of Zea$^{•+}$.[9] We did not observe any positive snapshot TA signal at 920 nm; therefore, it appears that Lut$^{•+}$ is not involved in this type of quenching in wild-type spinach thylakoids.

Using the same samples, we also conducted fluorescence lifetime snapshot experiments to evaluate Chl quenching behavior. In contrast to fluorescence yields, fluorescence decays of Chl are not dependent upon photobleaching or changes in Chl concentration, which allows for precise evaluation of NPQ originating from the activation of quenchers and related quenching mechanisms.[18−21] Figure 2a presents amplitude-weighted average lifetimes (τ_{avg}) and corresponding NPQ parameters (NPQτ) extracted from 68 fluorescence decays at 680 nm (Figure S2). NPQτ is a lifetime-based parameter analogous to the conventional NPQ value (= $(F_m - F'_m)/F'_m$),[18] and details of the calculation of NPQτ are presented in Experimental Methods section. When the actinic light was turned on, thylakoid samples showed a rapid decrease in Chl lifetime from 1.3 to 0.34 ns, eventually plateauing after 5 min, and 90% of maximum NPQτ was achieved within 3 min. This early time period (≤3 min) of NPQτ in response to light is similar to both the timing of the appearance of the maximum amount of Zea$^{•+}$ (Figure 1c) and the qE time scale.[1] Therefore, this set of data supports the idea that a Zea$^{•+}$-mediated CT quenching mechanism is a part of qE quenching.

To quantify the Zea conversion in spinach thylakoids, we performed HPLC measurements in parallel on the same batch of thylakoid samples at each time point. As shown in Figure 2b, the violaxanthin de-epoxidase (VDE) converted Vio to Zea exponentially (τ_{rise} = 2.66 min). To determine how much Zea is

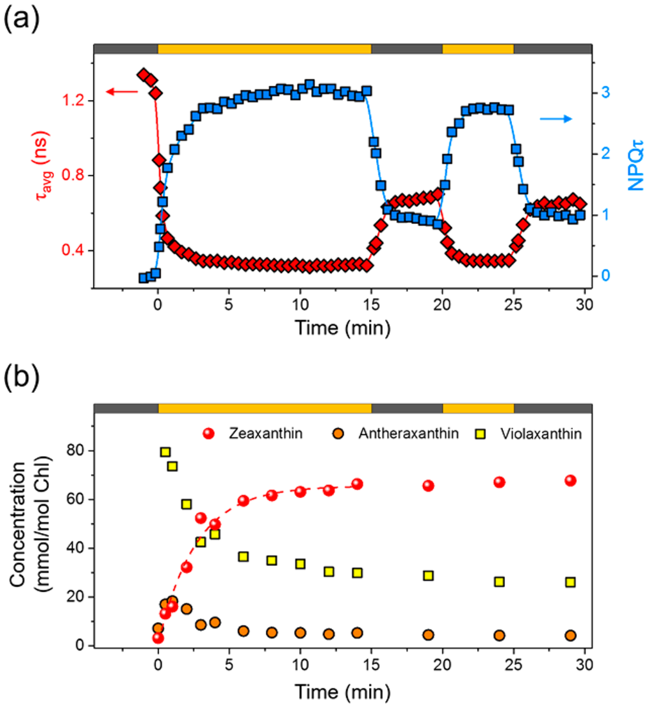


Figure 2. (a) Evolution of average Chl fluorescence lifetimes (τ_{avg}) in response to high light illumination and the NPQτ values (see text) calculated using corresponding τ_{avg} values at each time point. (b) Concentrations of Zea, antheraxanthin, and violaxanthin determined by time-resolved HPLC measurements. The dashed line indicates a single-exponential fit to Zea accumulation with a time constant of 2.66 min.

involved in CT quenching (or forms Zea$^{\bullet+}$), one needs to use the extinction coefficient (ε) of Zea$^{\bullet+}$. Unfortunately, there is a large variation in the values of ε for carotenoid radical cation reported previously, and none of these studies examined the value in a protein environment (see details in the Supporting Information).[22−25] Because we do not know the role of any protein-induced conformational changes on the ε, we decided to use a range of values for ε (53 000−73 000 L mol^{-1} cm^{-1}), centered around ε for β-carotene cation absorption at 970 nm (63 000 L mol^{-1} cm^{-1}) to quantify the Zea$^{\bullet+}$ represented in our TA data.[23] On the basis of these values of ε, we were able to estimate that after 1 min of light acclimation (maximum Zea$^{\bullet+}$ signal) only a small portion ($\leq$0.6%) of the Zea pool at that time was observed as the Zea$^{\bullet+}$ species in the snapshot TA signal. This is approximately equivalent to a Zea$^{\bullet+}$ in 5−7% of PSII supercomplexes (see the Supporting Information for values used in the calculation).[26−28] The bulk of the Zea may facilitate other quenching processes, such as LHCII migration or quenching of reactive oxygen species, as suggested previously.[16,29] Accordingly, we suggest that the Zea$^{\bullet+}$ formation is not simply proportional to the concentration of Zea but is rather controlled by ΔpH or ΔpH-triggered mechanisms such as activation of the PSII subunit S (PsbS) protein.[30]

It is well-accepted that qE mechanisms require activation of PsbS, which is initiated by ΔpH across the thylakoid membrane.[31−33] Recently, Correa-Galvis et al. reported a cross-linking assay using 3,3′-dithiobis-(sulphosuccinimidylpropionate) (DTSSP).[34] For dark-acclimated thylakoids, the DTSSP treatment arrests rearrangement and relocation of membrane proteins, preventing the protein—protein interactions required for qE activation. Although the DTSSP does not specifically target qE-specific PsbS interactions, it is tempting to speculate that the major effects of DTSSP are due to inactivated PsbS as the NPQ activation of DTSSP-treated thylakoid exhibits a very similar time course to that of the PsbS-deficient *npq4* mutant.[34] Therefore, we employed DTSSP treatment of our dark-acclimated thylakoid sample to examine the effect of chemical cross-linking on the CT quenching. In addition, we separately treated samples with 1,4-dithiothreitol (DTT), which is known to inhibit VDE activity and prevent conversion of Vio to Zea.[35,36]

Figure 3a shows the results of fluorescence lifetime snapshot measurements of DTSSP- and DTT-treated thylakoid samples.

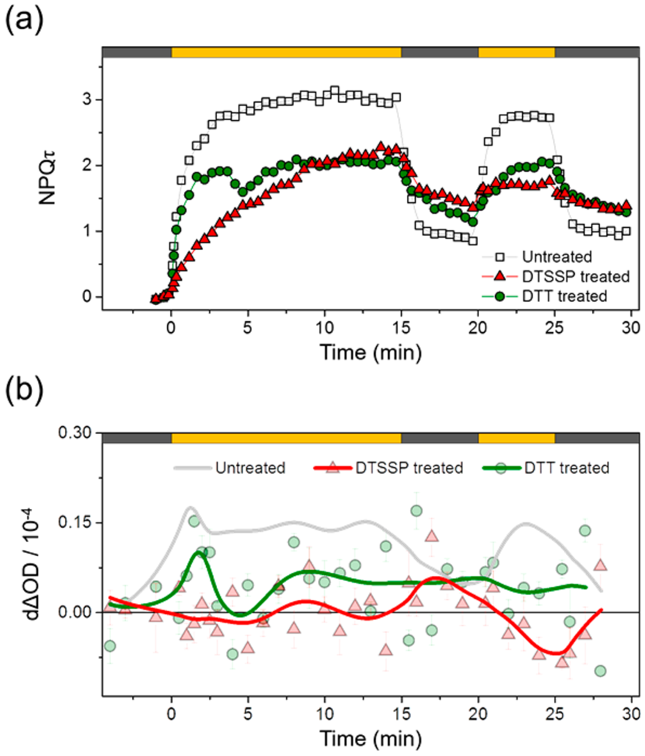


Figure 3. (a) Evolution of NPQτ values of DTSSP- and DTT-treated thylakoids in response to high light illumination. (b) Snapshot TA data of DTSSP- and DTT-treated thylakoids. The untreated sample is displayed with a smoothed trajectory (gray curve).

In the DTSSP-treated sample, it appears that the chemical cross-linking removed a qE component from the NPQ trace, resulting in slowed quenching and *npq4*-like (i.e., absence of PsbS) behavior.[20] Interestingly, DTSSP treatment had little impact on the activity of VDE and the rate of Zea formation (Figure S3a). The snapshot TA results in Figure 3b revealed that the DTSSP-treated thylakoids showed no significant Zea$^{\bullet+}$ in response to light, suggesting that rearrangement or conformational changes of active PsbS and partner proteins are necessary for Zea$^{\bullet+}$ formation.[6] This result supports the idea that the CT quenching mechanism is a part of NPQ quenching in higher plants and is triggered by a ΔpH → messenger proteins (e.g., PsbS) pathway[37,38] and/or the ΔpH and electric potential gradients ($\Delta\psi$) across the membrane stabilizing the state of CT quenching. In fact, the ΔpH obtained from the model of Zaks et al.,[37,38] the rate of quenching, and [Zea$^{\bullet+}$]/[Zea] are well-correlated, as shown in Figure 4a. This ΔpH-dependent CT quenching mechanism

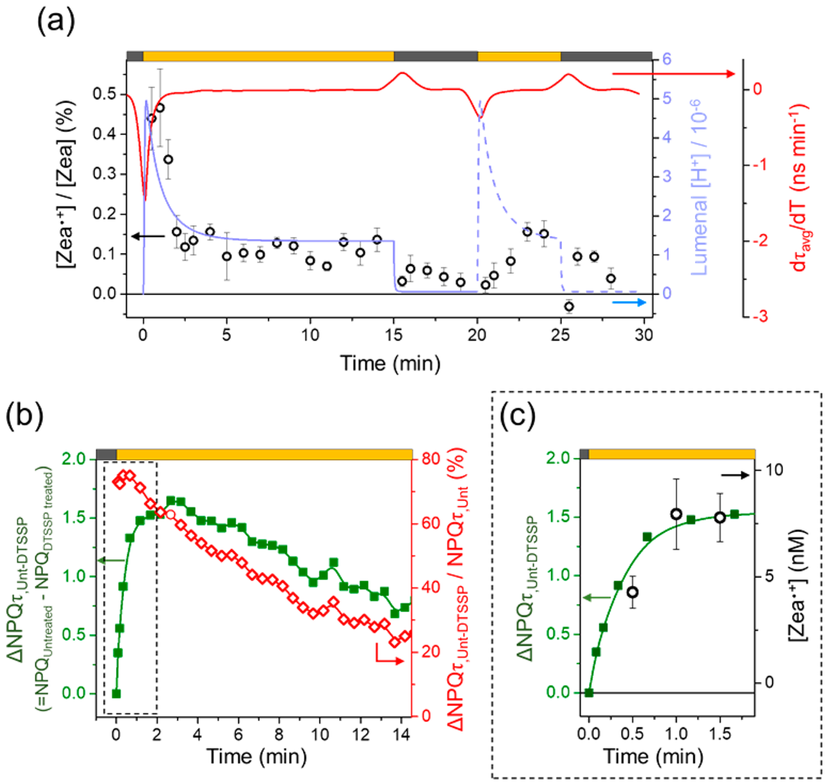


Figure 4. (a) Evolution of $[\mathrm{Zea}^{\bullet+}]/[\mathrm{Zea}]$ (circles), derivative of the fluorescence lifetime (τ_{avg}) (red line), and lumenal $[\mathrm{H}^+]$ (blue line) in response to high light/dark exposures. The data of lumenal $[\mathrm{H}^+]$ were calculated according to the kinetic model developed by Zaks et al.[37,38] The dashed blue line indicates significant uncertainty in lumenal $[\mathrm{H}^+]$ as the model was designed for light acclimation of completely dark-acclimated systems. (b) $\Delta\mathrm{NPQ}\tau_{,\mathrm{Unt-DTSSP}}$ (untreated minus DTSSP-treated samples) (green line with squares) and $\Delta\mathrm{NPQ}\tau_{,\mathrm{Unt-DTSSP}}$ as a percent of overall NPQ ($\mathrm{NPQ}\tau_{,\mathrm{Unt}}$) (red line with diamonds) during high-light-acclimation. (c) Rise of $\Delta\mathrm{NPQ}\tau_{,\mathrm{Unt-DTSSP}}$ (green line with squares) and $[\mathrm{Zea}^{\bullet+}]$ (circles) in the early (≤ 2 min) stage of high-light-acclimation. Note that the values of $[\mathrm{Zea}^{\bullet+}]$ were calculated based on an extinction coefficient of 63 000 L mol^{-1} cm^{-1}.[23]

resembles the pH dependency of quenching in other photosynthetic organisms such as *Physcomitrella patens*[39] and *Chlamydomonas reinhardtii*.[40]

If we make the assumption that DTSSP treatment only stops $\mathrm{Zea}^{\bullet+}$ formation but not Zea-dependent NPQ that does not involve $\mathrm{Zea}^{\bullet+}$ formation, the difference between the quenching behaviors (or decreases in fluorescence lifetime) of the untreated and the DTSSP-treated samples ($\Delta\mathrm{NPQ}\tau_{,\mathrm{Unt-DTSSP}}$) corresponds to the contribution of $\mathrm{Zea}^{\bullet+}$ formation to qE. In this case, the percentage of qE contributed by $\mathrm{Zea}^{\bullet+}$ formation is simply $\Delta\mathrm{NPQ}\tau_{,\mathrm{Unt-DTSSP}}/\mathrm{NPQ}\tau_{,\mathrm{Unt}}$. As Figure 4b shows, this percentage is as high as 75% in the first 2 min, falling to 25% by 15 min of high light exposure. Figure 4c shows that the initial rise in $\Delta\mathrm{NPQ}\tau_{,\mathrm{Unt-DTSSP}}$ is consistent with the rise of the $\mathrm{Zea}^{\bullet+}$ signal in the TA snapshot data. If the CT mechanism occurs only in the monomeric (minor) LHCII and the protonation of PsbS leads to dissociation of trimeric LHCIIs from the PSII supercomplexes,[15,16,34] the concomitant reduction in excitation of monomeric LHCIIs could account for the decrease of $\mathrm{Zea}^{\bullet+}$ contribution at longer light acclimation times. To be consistent with the snapshot TA data of the DTSSP-treated sample, the formation of $\mathrm{Zea}^{\bullet+}$ must also require dissociation of PsbS dimers or some other intermolecular rearrangement limited by DTSSP cross-linking. The present data, based on the assumptions above, suggest, but by no means prove, that CT quenching is one of the first responses to excess light, followed by a suite of processes with slower turn on times that involve Zea in a non-CT role,[8] Lut,[10] and possibly other actors.[41]

The DTT-treated thylakoids with inhibited VDE activity showed a lower level of maximum $\mathrm{NPQ}\tau$ ($\cong 2$), close to 2/3 the level of untreated samples. As presented in the HPLC data (Figure S3a), although there is a small increase in Zea concentration at 2 min after high light exposure, there was little to no increase by the end of the light sequence. However, the DTT-treated thylakoids still exhibited a moderate amount of TA signal at 1000 nm (Figure 3b). Considering that only a small portion of Zea is converted to $\mathrm{Zea}^{\bullet+}$, one possible explanation is that a pre-existing small pool of Zea ($\cong$3 mmol/mol Chl) is responsible for a large fraction of the CT quenching. It is also possible that antheraxanthin is involved in the quenching as early antheraxanthin levels were similar across untreated, DTT-treated, and DTSSP-treated thylakoids (Figure S3b).[37,42] It is noteworthy that a dip in the signal was observed at 5 min in both fluorescence and TA snapshot data. This suggests that the CT quenching is still important in the early stages of NPQ even with reduced VDE activity. In addition, as discussed above, it implies that the detachment of trimeric LHCIIs from the PSII supercomplex would transiently isolate them from the CT quenching sites on the monomeric LHCIIs.[15,16]

Our calculations indicate that $\leq$0.6% of Zea is responsible for the maximum $\mathrm{Zea}^{\bullet+}$ signal (Figure 4a), provided that our estimate of ε for $\mathrm{Zea}^{\bullet+}$ is roughly correct. This combined with our estimate that only 5−7% of PSII supercomplexes have $\mathrm{Zea}^{\bullet+}$ molecules present, assuming that $\mathrm{Zea}^{\bullet+}$ is only associated with the monomeric complexes, might be taken to imply a minor role of CT formation in qE. On the other hand, the identical rise of Zea$^{•+}$ and ΔNPQ$\tau_{\mathrm{,Unt-DTSSP}}$ suggests otherwise (Figure 4c). A possible resolution is suggested by the work of Walla and co-workers[43,44] and that of Dreuw et al.[45] Walla and co-workers show that NPQ onset is correlated with increased Car−Chl energy transfer in both directions,[43,44] while Dreuw et al. show that the nature of electronically excited states of Zea and Chl molecules in close proximity is very sensitive to the spatial separation of the pair.[45] If a range of Zea−Chl separations and a range of energy gaps exist in the thylakoid membrane, some will give energy transfer and some CT. In this scenario, the Zea$^{•+}$ could be taken as a marker of Zea−Chl interaction that leads to quenching via both CT and EET routes. We plan future snapshot TA spectroscopy studies to focus on other possible quenching mechanisms, including EET by measuring changes in absorption in the carotenoid S_1 wavelength region.[8] Snapshot TA studies of the wide variety of *Arabidopsis thaliana* photoprotection mutants available should greatly aid the exploration of NPQ mechanisms.

In summary, we have introduced snapshot TA spectroscopy, which allowed us to follow the appearance of a carotenoid radical cation signal as spinach thylakoid membranes acclimate to high light. We observed a maximum Zea$^{•+}$ signal after 2 min of light acclimation, consistent with observations from fluorescence lifetime measurements and with the time scale of qE. Time-resolved HPLC measurements revealed that Zea$^{•+}$ formation in spinach is not significantly limited by Zea accumulation, and the results of DTSSP and DTT treatments of the thylakoids suggest that Zea$^{•+}$ formation is highly dependent on reorganization or structural change of proteins, initiated by the pH-sensing protein, PsbS. Therefore, it seems likely that Zea is involved in a CT quenching mechanism in higher plants that rapidly responds to changes in light intensity, consistent with qE quenching.

■ EXPERIMENTAL METHODS

Preparation of Thylakoid Membrane. Fresh spinach leaves were acquired the day before preparation and dark acclimated at 4 °C overnight. Spinach thylakoid membranes were isolated by a modified version of the procedure described previously.[46] Crude thylakoids membranes were washed twice with 10 mL of suspension buffer before diluting with reaction buffer. The final concentration of all thylakoid samples was adjusted to 80 nmol Chl/mL before measurement. The working concentrations of DTSSP and DTT were 3 and 2 mM, respectively. The Zea accumulation in thylakoid samples was monitored by HPLC, as previously described.[47]

Snapshot Transient Absorption Spectroscopy. Pump−probe spectroscopy for TA measurements has been described in previous literature.[6,11] A Ti:sapphire oscillator (Coherent MIRA Seed) was used to seed a regenerative amplifier (Coherent RegA 9050) with an external stretcher/compressor, generating an 800 nm pulse with a repetition rate of 250 kHz. A portion of the pulses pumped an optical parametric amplifier (OPA, Coherent 9450) which was tuned to 650 nm for Chl b Q_y transition. We chose 650 nm as an excitation wavelength because the output power of our OPA was higher there than that at 680 nm (Chl a Q_y transition), yielding higher SN ratios. The pump pulses had a maximum pump energy of 40 nJ/pulse, and the fwhm of the autocorrelation trace was 54 fs. NIR continuum probe pulses were produced using a 1 mm yttrium aluminum garnet (YAG) crystal, and an 850 nm long-pass filter was placed after continuum generation. The polarizations of the pump and probe were set to the magic angle (54.7°) by placing a half wave plate and a polarizer in the pump path. The diameters of the pump and probe at the sample position were 120 and 73 μm, respectively. After the sample, a second polarization filter set to the probe polarization was placed to minimize pump scattering, as well as an 850 nm long pass filter to ensure a clean probe signal. A monochromator (SpectraPro 300i, Acton Research Corp., Action, MA) and an InGaAs photodiode (DET410, Thorlabs, Newton, NJ) were used to monitor the $\Delta T/T$ signal. The actinic light was set to an intensity of 850 μmol photons·m^{-2}·s^{-1} at the sample position, with a heat-absorbing filter (KG1). For collecting snapshot TA data at a fixed delay time (20 ps) and a wavelength (1000 nm), a pump and probe shutter was controlled to open for 10 s at 30 s−1 min intervals throughout the light acclimation sequence. The sample cell was moved between measurements to prevent sample damage. The path length of the cuvette was 1 mm.

Fluorescence Lifetime Snapshot. Fluorescence lifetime snapshot data was collected in a home-built fluorescence lifetime measurement apparatus previously described.[18−21] Briefly, the 840 nm output pulses with a repetition rate of 76 MHz from a Ti:sapphire oscillator (Coherent Mira 900f) were frequency-doubled to generate 420 nm using a beta barium borate (BBO) crystal, which is for excitation of the Soret band of Chl a. Before the sample, one portion was directed into a photodiode to provide SYNC for the time-correlated single-photon counting card (Becker-Hickl SPC-630 and SPC-850). The other portion of the laser was intermittently blocked by a shutter controlled by a LabVIEW program. The excitation laser power was 1.6 mW (21 pJ/pulse) at the sample. The sample was intermittently exposed to an actinic light (Schott KL1500) with an intensity of 850 μmol photons·m^{-2}·s^{-1}, also controlled by a shutter and LabVIEW program. After the sample, a monochromator set to 680 nm and a MCP PMT detector (Hamamatsu R3809U) were placed for fluorescence detection. The 68 fluorescence decay measurements were made at intervals varying from every 10 s to every 30 s in complete darkness. In each measurement, the sample was exposed to the laser for 1 s, divided up into five steps of 0.2 s. The step with the longest fluorescence lifetime was selected in data processing to ensure that the PSII reaction centers were closed. Each fluorescence decay curve was fit to a sum of three exponential decay components (Picoquant Fluofit Pro-4.6). Following data fitting, the amplitude-weighted average lifetime (τ_{avg}) and NPQτ values were calculated by the following equations[18,20]

$$\tau_{\mathrm{avg}} = \frac{\sum_i A_i \tau_i}{\sum_i A_i}$$

where A_i and τ_i are the amplitudes and the fluorescence lifetime components, respectively, and

$$\mathrm{NPQ}\tau = \frac{\tau_{\mathrm{avg,dark}} - \tau_{\mathrm{avg,light}}}{\tau_{\mathrm{avg,light}}}$$

where $\tau_{\mathrm{avg,dark}}$ is the average of three lifetimes measured at the initial dark period.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpclett.7b02486.

Detailed results of the reconstituted transient absorption spectrum, steady-state fluorescence emission spectrum, and time-resolved HPLC data (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Jonathan M. Morris, Eva M. Nichols, Dr. Lowell D. Kispert, Dr. Masakazu Iwai, and Dr. Tae Kyu Ahn for helpful discussions. This work was supported by the U.S. Department of Energy, Office of Science, Basic Energy Sciences, Chemical Sciences, Geosciences, and Biosciences Division under Field Work Proposal 449B. K.K.N. is an investigator of the Howard Hughes Medical Institute. S.P. is supported by the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education (NRF-2016R1A6A3A03006768). R.B. thanks the Miller Institute of Berkeley for awarding a visiting professorship grant.

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DOI: 10.1021/acs.jpclett.7b02486
J. Phys. Chem. Lett. 2017, 8, 5548−5554